JOSÉ BERNARDO ALMEIDA GARRETT DE NORONHA MATOS

ON THE ROLE OF P2 PURINOCEPTORS AND ECTO-NTPDASES IN POSTMENOPAUSAL HUMAN OSTEOGENESIS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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ABBREVIATIONS

3'-UTR, Three prime untranslated region

25-OH-Vitamine D, 25-OH-Cholecalciferol

a.u., Arbitrary units

A438079, 3-[[5-(2,3-Dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine hydrochloride

ADO, Adenosine

ADPβS, Adenosine 5'-[β-thio]diphosphate

ALP, Alkaline phosphatase

AMP, Adenosine 5'-monophosphate

AP-1, Adapter-related protein complex 1 subunit

AR, Androgen receptor

ARL 67156, 6-*N*,*N*-Diethyl-D-β,γ-dibromomethylene ATP trisodium salt

ATP, Adenosine 5'-triphosphate

ATPase, Adenosine 5'-triphosphatase

***BGLAP*,** Osteocalcin coding gene

BMP, Bone morphogenic protein

BMPRIA, Bone morphogenic protein receptor type IA

BMSCs, Bone marrow stromal cells

BRU, Bone remodelling units

BTE, Bone tissue engineering

BzATP, 2'(3')-*O*-(4-Benzoylbenzoyl)adenosine 5'-triphosphate

$[Ca^{2+}]_i$, Intracellular calcium

cAMP, 3'-5'-Cyclic adenosine monophosphate

CaSR, Calcium sensing receptors

CD11b, Integrin alpha M

CD14, Monocyte differentiation antigen CD14

CD19, B-lymphocyte antigen CD19

CD34, Haematopoietic progenitor cell antigen CD34

CD39, Apyrase or NTPDase1

CD44, Receptor for hyaluronic acid

CD45, Protein tyrosine phosphatase, receptor type C, also known as PTPRC

CD49, Integrin alpha subunit or very late antigen

CD54, Intercellular adhesion molecule -1

CD73, Ecto-5'-nucleotidase

CD79, B-cell antigen receptor complex-associated protein (alpha or beta) chain

CD90, Thy-1 membrane glycoprotein

CD105, Endoglin

CD164, Sialomucin-like 2 protein

c-Fos, G0/G1 switch regulatory protein 7 or Proto-oncogene *c-Fos*

CHL, chelerythrine

c-Jun, Transcription factor AP-1

c-Kit, Tyrosine-protein kinase Kit

***COL1A1*,** Collagen alpha-1(I) chain coding gene

COL1A2, Collagen alpha-2(I) chain coding gene

CR, Calcitonin receptor

CREB, cAMP response element-binding protein

CXCR, chemokine receptors

CYP27B1, 1α-hydroxylase

DAG, Diacylglycerol

DNA, Deoxyribonucleic acid

ECM, Extracellular matrix

ER, Oestrogen receptor

ERK, Extracellular signal-regulated kinase

ESC, Embryonic stem cells

FACS, Fluorescence-activated cell sorting

FAK, Focal adhesion kinase

FBS, Foetal bovine serum

FGF, Fibroblast growth factor

FOSB, Fos related factor B

FRA, Fos related antigen

GAG, Glycosaminoglycan

G-CSF, Granulocyte colony stimulating factor

Gla, γ-Carboxyglutamic acid

GPCR, G-protein-coupled receptor

H, Hour

H1152, (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl) sulfonyl]-hexahydro-1*H*-1,4-diazepine

HLA-DR, HLA class II histocompatibility antigen gamma chain

HPLC, High-performance liquid chromatography

HSCs, Haematopoietic stem cells

HSPC, Haematopoietic stem progenitor cells

***IBSP*,** Integrin-binding sialoprotein coding gene

ICAM, intracellular cell adhesion molecule

IFN, Interferon

IGF, Insulin growth factor

IL, Interleukin

IP3, Inositol trisphosphate

JNK, c-Jun NH2-terminal protein kinase

JunB, Transcription factor jun-B

JunD, Transcription factor jun-D

LPA, Lysophosphatidic acid

MAPK, Mitogen-activated protein kinase

M-CSF, macrophage colony stimulating factor

MEK, Dual specificity mitogen-activated protein kinase kinase

Micro-CT, Microtomography or computed tomography

Min, Minute

miRS, Small non-coding ribonucleic acid molecule

mRNA, Messenger ribonucleic acid

MRS 2179, 2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt

MRS 2578, *N*,*N*''-1,4-Butanediyl*bis*[*N*'-(3-isothiocyanatophenyl)thiourea

MSCs, Mesenchymal stem cells

MTT, 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide

Nanog, Homeobox transcription factor Nanog

NDPK, Nucleoside diphosphokinase

NECA, 5'-(N-ethylcarboxamido)adenosine

NFATc1, Nuclear factor of activated T-cells, cytoplasmic 1

NIK, NF-κB-inducing kinase

NK, Natural killer

Notch, Neurogenic locus notch homolog protein

NPP, Nucleotide pyrophosphatase/ phosphodiesterase

NR3C1, Nuclear receptor subfamily 3, group C, member 1 or glucocorticoid receptor

NTPDase, Nucleoside triphosphate diphosphohydrolase

OCPs, Osteoclast precursors

Oct-3/4, POU domain, class 5, transcription factor 1

OPG, Osteoprotegerin

OSE, Osteoblast specific element

OSX, Osterix

p53, Tumor suppressor p53

$p66^{shc}$, SHC (Src homology 2 domain containing) transforming protein 1 isoform $p66^{Shc}$

PGE_2, Prostaglandin E_2

Pi, Inorganic phosphate

PIP5K1, Phosphatidylinositol-4-phosphate 5-kinase type I

PKA, Protein kinase A

PKC, Protein kinase C

PLA_2, Phospholipase A_2

PLC, Phospholipase C

PLD, Phospholipase D

PMA, phorbol 12-myristate 13-acetate

PNP, *p*-Nitrophenol

POM 1, Sodium metatungstate

PPADS, Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid

PPi, Pyrophosphate

PSB 0474, 3-(2-Oxo-2-phenylethyl)-uridine-5'-diphosphate disodium salt

PSB 603, 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine

PTH, Parathyroid hormone

PTHR, Parathyroid hormone receptor

Raf, Ras-binding domain

RANK, Receptor activator of NF-κB

RANKL, Receptor activator of NF-κB ligand

Ras, Member of protein superfamily of small GTPases

Rex-1, Zinc finger protein 42 homolog

RNA, Ribonucleic acid

RTK, receptor tyrosine kinase

Runx-2, Runt-related transcription factor 2

SBE, Smad binding elements

Sca-1, Spinocerebellar ataxia type 1 or Ataxin-1

SCF, Stem cell factor

SDF, Stromal derived factor

Sec, Second

SERM, Selective oestrogen receptor modulators

SIBLINGS, Small Integrin-binding Ligand, N-linked Glycoproteins

siRNA, Small interfering ribonucleic acid

Smad, Mothers against decapentaplegic homolog

***SP7*,** Osterix coding gene

***SPARC*,** Osteonectin coding gene

***SPP1*,** Osteopontin coding gene

TERC, Telomerase ribonucleic acid component

TERT, Telomerase reverse transcriptase

TGF, Transforming growth factor

TNAP, Tissue non-specific alkaline phosphatase

TNF, Tumour necrosis factor

TO-PRO-3, Quinolinium 4-[3-(3-methyl-2(3*H*)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-diiodide

TRAFs, Tumour necrosis factor receptor-activating factors

TRPV, Transient receptor potential cation channel subfamily V member

U0126, (2Z,3Z)-2,3-bis(Amino(2-aminophenylthio)methylene)succinonitrile

U73122, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione

UDP, Uridine 5’-diphosphate

UMP, Uridine 5’-monophosphate

US, Ultrasound

UTP, Uridine 5’-triphosphate

UTPγS, Uridine-5'-(γ-thio)-triphosphate

VCAM, Vascular cell adhesion protein

VDR, vitamin D receptors

VLA, very late antigen

Yr, Years

RESUMO

O tecido ósseo é um tecido conjuntivo especializado que contém células (osteoblastos, osteoclastos, osteócitos) e minerais depositados numa matriz orgânica de colagénio. A remodelação contínua do osso possibilita a sua reparação, crescimento e adaptação. Numa vida, muitas anomalias podem levar a alterações neste processo que é controlado de modo fino, resultando numa grande diversidade de doenças ósseas e anomalias esqueléticas (como a osteoporose). O desenvolvimento de novas terapias para as doenças ósseas é de grande relevância visto que muitas destas alterações têm uma crescente prevalência na população humana envelhecida.

A remodelação óssea é um processo complexo que é regulado de forma apertada. Para além do envolvimento de fatores sistémicos, intervêm diversos fatores locais, tais como o 5'-trifosfato de adenosina (ATP) e os seus derivados (como a adenosina). Foi demonstrado que células ósseas osteoprogenitoras (como as células mesenquimatosas) e os osteoblastos libertam constitutivamente nucleótidos (como por exemplo o ATP) quando são sujeitas a estímulos mecânicos ou em situações patológicas, como a hipoxia e a inflamação. Os nucleótidos podem ser libertados por vários mecanismos: (1) lesão celular e tecidular (incluindo locais de lesão óssea), (2) por exocitose e (3) por intermédio de canais ou poros membranares na ausência de lise celular, incluindo neste último mecanismo as junções estreitadas. Uma vez libertados, os nucleótidos e seus derivados podem ativar recetores purinérgicos que são importantes moduladores da formação óssea, embora a sua ação seja ainda amplamente desconhecida. Os recetores para as purinas e pirimidinas podem ser classificados em dois tipos: recetores P1 (A_1, A_{2A}, A_{2B} e A_3), que são ativados pela adenosina, e recetores P2, ativados pelos nucleótidos de adenina e uracilo (*e.g.* ATP, ADP, UTP, UDP). Estes podem ser subdivididos em recetores P2X ionotrópicos (canais iónicos ativados por ligandos) e recetores P2Y metabotrópicos (acoplados a proteínas G). A ativação dos recetores purinérgicos é balanceada pela atividade de ecto-nucleotidases expressas na membrana das células, que promovem o metabolismo extracelular dos nucleótidos, levando à formação dos respetivos nucleósidos 5'-di- e mono-fosfatos, nucleósidos, fosfatos e pirofosfatos.

Existem evidências de que a sinalização purinérgica exerce um efeito local complexo nas células ósseas. Contudo, existe uma enorme controvérsia em torno da importância de cada recetor purinérgico e das enzimas metabolizadoras dos nucleótidos, bem como em saber de que modo a interação entre estes intervenientes promove a remodelação óssea. Infelizmente, muitos dos estudos derivam da utilização de modelos animais e do uso de células humanas imortalizadas, sendo ainda escassos os trabalhos realizados em células humanas não modificadas (osteoprogenitoras e osteoblastos).

Visto que a sinalização purinérgica tem sido associada a diferentes patologias ósseas, torna-se imprescindível o seu estudo na tentativa de clarificar os mecanismos moleculares responsáveis pela atuação dos nucleótidos e nucleósidos na remodelação óssea em situação fisiológica e na doença. O sucesso deste estudo poderá resultar na descoberta de novos alvos terapêuticos para as patologias ósseas. Este projeto foi delineado para avaliar a expressão diferencial dos recetores P2 nas células humanas osteoprogenitoras / osteoblastos atendendo à idade e género dos doentes. Foi, ainda, decidido documentar as respostas funcionais destas células na presença de nucleótidos no meio extracelular e investigar o envolvimento de alguns subtipos de recetores P2 na libertação de ATP e na plasticidade membranar responsável pelos fenómenos de diferenciação/proliferação celular. Devido à sua relevância no controlo da atividade purinérgica, estudou-se a influência das NTPDases na proliferação, diferenciação e mineralização das células osteoprogenitoras. Especificamente, neste projeto investigou-se: **(1)** a expressão e o papel funcional dos recetores sensíveis a nucleótidos de uracilo ($P2Y_2$, $P2Y_4$, e $P2Y_6$) em células osteoprogenitoras / osteoblastos, visto que o seu papel na diferenciação osteogénica era desconhecido; **(2)** a expressão e função dos recetores ionotrópicos P2X7 na osteogénese humana, explorando os mecanismos moleculares envolvidos no sentido de esclarecer alguma controvérsia sobre o papel destes recetores existente na literatura, no que toca às células humanas; **(3)** a importância das NTPDases na proliferação e diferenciação das células osteoprogenitoras da medula óssea em mulheres jovens e com idade pós-menopáusica.

As amostras de medula óssea foram obtidas de mulheres pós-menopáusicas sujeitas a artroplastia da anca como resultado de osteoporose primária. Para comparação, foram obtidas amostras da medula óssea proveniente de mulheres jovens sujeitas a colheita de enxerto ósseo para correção cirúrgica de escoliose ou fraturas traumáticas. O isolamento das células estromais mesenquimatosas foi demonstrado por análise imunofenotípica por citometria de fluxo; estas células apresentavam marcadores encontrados caracteristicamente em células mesenquimatosas multipotentes da medula óssea, nomeadamente os marcadores CD105, CD73, CD117 e CD29, não apresentando marcação para células estaminais hematopoiéticas, nomeadamente CD14 e CD45.

Relativamente ao objetivo **(1)**, verificou-se que a incubação das células estromais mesenquimatosas da medula óssea (MSCs) provenientes de mulheres pós-menopáusicas com UTP ou UDP promove a sua diferenciação osteogénica, traduzindo-se num aumento de atividade da fosfatase alcalina (ALP) para níveis semelhantes aos observados em mulheres jovens, sem contudo se observar qualquer efeito na proliferação celular. Os nucleótidos de uracilo aumentam de forma dependente da concentração os níveis de cálcio intracelular ($[Ca^{2+}]_i$) em MSCs. No entanto, estes efeitos tornam-se menos evidentes com o tempo das células em cultura (7>21 dias). A ativação seletiva de recetores $P2Y_6$ com o análogo estável do UDP, PSB 0474, mimetizou os efeitos do UTP e do UDP, enquanto o análogo estável do UTP, UTPγS, foi desprovido de efeito. O antagonista seletivo do recetor $P2Y_6$, MRS 2578, preveniu o aumento dos níveis intraceulares de $[Ca^{2+}]_i$ e a diferenciação osteogénica provocada pelo UDP em todos os períodos de cultura analisados. As MSCs demonstram imuno-reatividade para os recetores $P2Y_2$, $P2Y_4$, e $P2Y_6$. Enquanto a expressão do recetor $P2Y_6$ se mantém constante ao longo do período de cultura (7~21 dias), a expressão dos recetores $P2Y_2$ e $P2Y_4$ é mais evidente em células mais diferenciadas (7<21 dias). O catabolismo extracelular dos nucleótidos de uracilo, UTP e UDP, foi maior em células menos proliferativas e mais diferenciadas (7<21 dias), que é justificado pelo aumento da expressão das NTPDases1, -2 e -3 nas populações de células mais diferenciadas (7<21 dias).

No que concerne ao objetivo **(2)**, verificámos que o ATP e o agonista do recetor P2X7 aumentam os níveis intracelulares de $[Ca^{2+}]_i$ em MSCs, paralelamente à formação de poros membranares permeáveis à sonda TO-PRO-3. A ativação dos recetores P2X7 promove a formação reversível de microvesículas na membrana plasmática e a formação de bolhas (zeiose) em MSCs. Apesar da diferenciação osteogénica promovida pelas alterações na dinâmica membranar ser independente do $[Ca^{2+}]_i$, ela parece envolver a atividade da PLC (fosfolipase C), da PKC (proteína cinase C) e da Rho-cinase secundárias à ativação do recetor P2X7. O agonista P2X7, BzATP, antecipa a diferenciação osteogénica (aumento da atividade ALP e da expressão dos fatores de transcrição Runx-2 e Osterix) e promove a mineralização das culturas de MSCs.

Quanto ao objetivo **(3)**, demonstrámos que o catabolismo extracelular dos nucleótidos de adenina e uracilo leva a uma perda da atividade dos recetores $P2Y_6$ e P2X7 em MSCs de mulheres pós-menopáusicas quando comparado com as mulheres jovens. Verificou-se que a expressão da NTPDase3 aumenta com o tempo das células em cultura nas mulheres pós-menopáusicas, sendo que esta enzima está ausente em MSCs de mulheres jovens. A inibição seletiva da NTPDase3 com o fármaco, PSB 06126, aumenta 3.6 vezes os níveis endógenos de ATP em culturas de MSCs de mulheres pós-menopáusicas avaliadas ao dia 7. A inibição da NTPDase3 favorece a atividade da ALP e promove a expressão de marcadores de osteogénese, Runx-2 e Osterix, ao longo do tempo de cultura. É, ainda, de realçar que a inibição da atividade da NTPDase aumentou de forma significativa ($P<0.05$) a formação de nódulos de mineralização nas culturas de MSCs comparativamente as mesmas culturas na ausência dos inibidores. Este efeito positivo na osteogénese foi totalmente prevenido na presença de apirase (CD39), a enzima que converte nucleótidos de adenina (ATP) e uracilo (UTP) nos seus derivados monofosfatados, AMP e UMP respectivamente. O antagonismo seletivo de recetores P2X7 e $P2Y_6$ atenuou ($P<0.05$) de igual forma os efeitos dos inibidores da NTPDase3 na osteogénese.

Concluindo, neste trabalho demonstra-se que os nucleótidos de uracilo são importantes reguladores da diferenciação de células osteoprogenitoras, predominantemente por intermédio da ativação de recetores $P2Y_6$ sensíveis ao

UDP por um mecanismo associado ao aumento dos níveis de $[Ca^{2+}]_i$. A ação endógena dos nucleótidos de uracilo pode ser balanceada por NTPDases específicas que irão determinar se as células osteoprogenitoras se diferenciam ou proliferam; verificou-se, ainda, que o recetor P2X7 é um importante regulador da diferenciação osteogénica e da consequente mineralização das MSCs humanas em cultura. Os mecanismos dependentes da ativação P2X7 podem envolver oscilações do $[Ca^{2+}]_i$ e alterações na dinâmica membranar (formação de poros e de bolhas) por mecanismos dependentes da atividade da PLC, PKC e Rho-cinase; estes processos controlam a expressão de marcadores envolvidos na diferenciação osteogénica, tais como o Runx-2 e o Osterix. Demonstrou-se, ainda, que a expressão da NTPDase3 controla a atividade dos recetores P2 nas MSCs em mulheres pós-menopáusicas. A expressão da NTPDase3 parece aumentar com a idade, facto que pode contribuir para reduzir dos níveis endógenos de nucleótidos de adenina e de uracilo na medula óssea das mulheres pós-menopáusicas e, deste modo, a ativação de importantes promotores da osteogénese, nomeadamente os recetores P2X7 e $P2Y_6$.

O conhecimento de alvos reguladores da diferenciação osteogénica anteriormente ignorados permitem agora o desenvolvimento de novas estratégias terapêuticas para controlar as doenças ósseas em que a erosão se sobrepõe à formação de novo osso, tais como a osteoporose, a artrite reumatoide e osteogénese imperfecta.

ABSTRACT

Bone is a specialized connective tissue, containing cells (osteoblasts, osteoclasts and osteocytes) and inorganic mineral salts deposited within an organic collagen matrix. The continuous remodelling of the bone allows it to repair, to grow and adapt. Throughout life, many abnormalities in such balanced process may result in a huge variety of bone disorders and skeletal abnormalities (such as osteoporosis). The development of new therapeutic approaches is of major importance since some bone disorders are highly prevalent and in increasing incidence on an ageing population.

Bone turnover is a complex and finely tuned process. To the importance of systemic factors that regulate bone turnover, we must include the role of local factors such as adenosine 5'-triphosphate (ATP) and its derivatives (such as adenosine). It was previously found that bone cells, such as osteoprogenitors (mesenchymal stem cells, MSCs) and osteoblasts constitutively release nucleotides (*e.g*. ATP) when submitted to mechanical stress or under pathological conditions, such as hypoxia and inflammation. Nucleotides may be released into the extracellular milieu through distinct mechanisms: (1) cytosolic release of ATP from sites of tissue and cell damage (including sites of bone injury), (2) exocytic release and (3) via intrinsic plasma membrane channels or pores in the absence of cytolysis, which includes hemichannels controlled release. Once released, nucleotides and their derivatives may act on purinoceptors, which are important (yet largely unknown) bone turnover regulators. The receptors for purines and pyrimidines are classified into two groups: P1 receptors (A_1, A_{2A}, A_{2B}, A_3), which are primarily activated by adenosine, and P2 receptors, which respond to adenine and uracil nucleotides (*e.g.* ATP, ADP, UTP, UDP). The latter may be further subdivided into P2X ligand-gated ion channels and P2Y G-protein-coupled receptors. Purinoceptors activation may be terminated by ecto-nucleotidases and other ecto-phosphatases bound to the plasma membrane, which rapidly hydrolyse extracellular nucleotides to their respective nucleoside 5'-di- and mono-phosphates, nucleosides and free phosphates or pyrophosphates.

Current evidence shows that purinergic signalling exerts complex local effects in bone microenvironment. However, controversy still exists around the functional relevance of each purinergic receptor and enzyme on bone cells and how they may interact to promote bone remodelling. In this regard, most of the studies derive from using animal models and immortalized cell lines and less in non-modified human cells (osteoprogenitors / osteoblasts).

Since purinergic signalling has now been implicated in many bone disorders, it is of great importance to explore these potential targets for future therapies, clarifying the molecular mechanisms operating upstream and downstream receptors activation in both health and disease conditions. To this end, this project was designed to evaluate changes in the expression of P2 receptors in human osteoprogenitors / osteoblasts taking into consideration age and gender of the patients, to document functional responses to extracellular nucleotides, to investigate the involvement of P2 receptors on ATP release and cell-membrane plasticity (required for osteoblast proliferation/differentiation), and study the impact of NTPDases in such mechanisms. Specifically, this project aimed at investigating: **(1)** the expression and function of uracil nucleotide-sensitive receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) in osteoprogenitors / osteoblasts, since their role in osteogenic differentiation was largely unknown; **(2)** the expression and function of P2X7 receptors on osteogenic differentiation of osteoprogenitors / osteoblasts in culture, exploring the molecular mechanisms involved; in literature, the role of the human P2X7 receptor is still controversial; **(3)** the role of the activity of NTPDases in the management of cell differentiation and/or proliferation, in both health and disease conditions; this topic is, so far, largely unknown particularly in non-modified human osteoprogenitor cells.

Bone marrow specimens were obtained from postmenopausal female patients undergoing total hip arthroplasty as a result of primary osteoarthrosis. For comparison purposes, it was also used bone marrow from younger female patients requiring bone engraftment for spinal fusion to correct scoliosis or to repair traumatic bone fractures. Isolation of MSCs was proven by immunophenotypic analysis (flow cytometry), showing positivity for markers of bone marrow-derived mesenchymal stromal cells, namely CD105, CD73, CD29 and CD117, in the

absence of the expression of haematopoietic stem cell markers, such as CD14 and CD45.

Regarding goal **(1)**, it was demonstrated that the application of UTP and UDP promotes osteogenic differentiation of postmenopausal bone marrow MSCs in culture measured as increases in alkaline phosphatase (ALP) activity to levels which are observed in younger patients, with no effects on cell proliferation. Uracil nucleotides concentration-dependently increase intracellular calcium ($[Ca^{2+}]_i$) in MSCs; their effects become less evident with the time (7>21 days) in culture. Selective activation of $P2Y_6$ receptors with the stable UDP analogue, PSB 0474, mimicked the effects of both UTP and UDP, whereas UTPγS was devoid of effect. Selective blockade of $P2Y_6$ receptors with MRS 2578 prevented $[Ca^{2+}]_i$ rises and osteogenic differentiation caused by UDP at all culture time points. MSCs are immunoreactive against $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. While the expression of $P2Y_6$ receptors remains fairly constant (7~21 days), $P2Y_2$ and $P2Y_4$ become evident only in less proliferative and more differentiated cultures (7<21 days). The rate of extracellular UTP and UDP inactivation was higher in less proliferative and more differentiated cell populations. It was also found that immunoreactivity against NTPDase1, 2, and 3 raises as cells differentiate (7<21 days).

Concerning the objective **(2),** ATP, and the P2X7 receptor agonist, BzATP, increased $[Ca^{2+}]_i$ in parallel to the formation of TO-PRO-3 permeable membrane pores. The two P2X7 agonists elicited reversible cell microvesiculation and plasma membrane blebbing (zeiosis). Differentiation-inducing plasma membrane dynamics was Ca^{2+}-independent, but involved the PLC (phospholipase C), PKC (protein kinase C) and Rho-kinase pathway downstream P2X7 receptor activation. BzATP anticipated osteogenic differentiation (increases in ALP activity and in the expression of Osterix and Runx-2 transcription factors) and favoured mineralization of MSC cultures.

Regarding goal **(3)**, it was found that that enzymatic inactivation of extracellular nucleotides leads to a loss of function of $P2Y_6$ and P2X7 purinoceptors in MSCs from postmenopausal women as compared to younger females. Interestingly, the expression of NTPDase3 increased with the culture time of MSCs from postmenopausal women; this enzyme was not expressed in the cells from younger

female patients. Selective inhibition of NTPDase3 with PSB 06126 increased by 3.6-fold the endogenous levels of ATP in MSC cultures from postmenopausal woman at day 7. NTPDase3 inhibition also increased the ALP activity and promoted the expression of osteogenic markers, both Runx-2 and Osterix, throughout the culture period. Under these circumstances, mineralization of bone-nodules increased (P<0.05) when compared to the control situation at culture day 43. The osteogenic differentiating effect of NTPDase3 inhibition was fully prevented by apyrase (CD39), the enzyme that converts directly nucleoside trisphosphates into their monophosphate derivatives. Selective blockade of P2X7 and $P2Y_6$ receptors also attenuated (P<0.05) the osteogenic effect of NTPDase3 inhibitors.

In conclusion, we demonstrated in this study that uracil nucleotides are important regulators of osteogenic differentiation of MSCs via the activation of UDP-sensitive $P2Y_6$ receptors coupled to increases in $[Ca^{2+}]_i$. The endogenous actions of uracil nucleotides may be balanced through the action of specific NTPDases determining whether osteoblast progenitors are driven into proliferation or differentiation. In addition, data show that the P2X7 receptor is an important regulator of osteogenic differentiation and subsequent mineralization of human MSCs in culture. The mechanisms by which the P2X7 receptor control bone formation might involve intracellular $[Ca^{2+}]_i$ oscillations and membrane cell dynamics (pore formation and blebbing) due to downstream activation of PLC, PKC and Rho-kinase-dependent pathways controlling osteogenic markers, like Runx-2 and Osterix. Here, we show for the first time that NTPDase3 is an important modulator of the activity of P2 purinoceptors in MSCs from postmenopausal woman. Age-related overexpression of NTPDase3 may contribute to reduce the endogenous amounts of adenine and uracil nucleotides, thus compromising the activation of important inducers of osteogenesis, such as P2X7 and $P2Y_6$ receptors.

These previously unrecognized targets for local regulation of osteogenic differentiation of bone marrow MSCs may prompt for novel therapeutic strategies to control human ossification disorders where bone destruction exceeds bone formation (*e.g.*, osteoporosis, rheumatoid arthritis, osteogenesis imperfecta).

1. INTRODUCTION

1.1. Stem cells and the stem cell niche

1.1.1. Adult stem cells – an introduction

Stem cells are the foundation cells for every organ and tissue in the body. Stem cells are cells with the ability to grow and to differentiate into more than 200 cell types. These are able to divide and to give rise to identical daughter cells (known as symmetrical division) and to differentiate into specific cells of somatic tissues (Avery et al., 2006). Stem cells may be found in: early embryos (commonly known as embryonic stem cells) and adult tissues (known as adult stem cells).

Stem cells can be classified with respect to their potency, that is, the competency of each cell to differentiate into a specialized type of tissue cells of the body. There are five class of potency for stem cells: totipotent, pluripotent, multipotent, oligopotent and unipotent (see *e.g*., Kaveh et al., 2011). Totipotent stem cells are able to give rise to all embryonic somatic cells and germ cells; they can generate a viable embryo (including extraembryonic support tissues such as the placenta). These cells result from the fusion of an ovum and sperm cell. Cells that result from the 1st divisions of the fertilized egg are totipotent cells (Volarevic et al., 2011). Pluripotent cells descend from totipotent cells and can give rise to cells of the three germ layers: endoderm, mesoderm, and ectoderm; they have no contribution to extraembryonic membranes or the placenta. Multipotent cells give rise to cells of a particular lineage or closely related family (Behr et al., 2010), that is, they can differentiate into a number of cells but from a germ layer. Oligopotent stem cells can differentiate into a few specialized cells and unipotent cells are limited to one cell type, although with the ability of self-renewal (Mitalipov and Wolf, 2009). Stem cells are part of an individual entire lifetime. Embryonic stem cells are the cells that are derived from the inner cell mass of an early stage of the embryo known as blastocyst (which consists of 50-150 cells, after 4-5 days post fertilization). Adult stem cells reside in most mammalian tissues, and the extent to which they contribute to normal homeostasis and repair is widely variable. Stem

cells have been found in many tissues and organs including epidermis, liver and bone (Kao et al., 2008). It is believed that the main role of adult stem cells is to replace damaged and injured tissues (Volarevic et al., 2011).

Among adult stem cells, we can find the haematopoietic stem cells (HSCs), epithelial stem, muscle stem, neural stem and mesenchymal stem cells (MSCs) (Kao et al., 2008; Kaveh et al., 2011). In the bone marrow reside mainly HSCs and MSCs. HSCs are the blood-forming cells, that is, they are ultimately responsible for the constant renewal of blood with the production of billions of new blood cells each day. They can also be found in cord blood, foetal liver, adult spleen, and peripheral blood. Mature haematopoietic cells are traditionally categorized into two distinct lineages: the lymphoid and the myeloid. The lymphoid lineage consists of T, B and natural killer (NK) cells, while the myeloid lineage includes a number of morphologically, phenotypically and functionally distinct cell types such as different subclasses of granulocytes (neutrophils, eosinophils and basophils), monocytes–macrophages, erythrocytes, megakaryocytes and mast cells (for a review, see Iwasaki and Akashi, 2007). HSCs usually express surface markers that can be used for their identification, namely Sca-1, CD14, CD34 and CD45. MSCs are multipotent, self-renewable cells that can be found not only in the bone marrow, but also in all postnatal organs and tissues, namely adipose tissue, umbilical cord blood and compact bone (Porada et al., 2006; Volarevic et al., 2010). These are a rare-population of non-haematopoietic stromal cells, able to differentiate into mesenchymal tissues such as bone, cartilage, adipose tissue and muscle (Augello et al., 2010). They are also able to differentiate into non-mesenchymal cells such as neurons (Kopen et al., 1999). In addition, they have a high proliferative capability, while retaining their undifferentiated state (Banfi et al., 2002; Bruder et al., 1997). MSCs show variable levels of expression of several markers, namely CD105 (SH2), CD90, CD73 (or ecto 5'-nucleotidase), stromal antigen 1, CD44, CD166 (vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule), CD49 and c-Kit (or CD117) (Baddoo et al., 2003; Boiret et al., 2005; Cognet and Minguell, 1999; Dennis et al., 2002; Gronthos et al., 2003; Pittenger et al., 1999; Sivasubramaniyan et al., 2012). However, some variations may be found

depending on the species, the tissue from where they were isolated and, of course, due to the *ex vivo* manipulations (Augello et al., 2010; Augello and De Bari, 2010; Jones et al., 2002). MSCs are able to leave their bone marrow niche and circulate through the bloodstream, assisting in repair of tissues when required (Bobis et al., 2006).

Adult stem cells have captured attention with the promise of tissue repair and the treatment of degenerative diseases, among others. The challenge is to stimulate stem cells *in vivo* or *in vitro* to develop various numbers of specialized cells that may represent sources of cells suitable for transplantation in a cell-based therapy. Hopefully these may be used to treat genetic and degenerative diseases, among others (Volarevic et al., 2011).

1.1.2. Adult stem cells and therapy – summary of promising applications in the context of bone tissue engineering

As mentioned earlier, MSCs are able to differentiate into osteoblasts, the bone forming cells (Friedenstein et al., 1966; Luria et al., 1987). The possibility to use such cells in bone repair in known bone disorders, such as osteoporosis and rheumatoid arthritis, has been proposed previously. In particular, bone tissue engineering (BTE) has emerged as a promising field. BTE refers to the implantation of a scaffolds seeded with appropriate number of cells, plus growth factors at the bone defect site. The cells and the scaffold itself are the two key points in BTE. Bone marrow mesenchymal stem cells are a type of cells usually chosen in BTE in order to reconstruct bone defects since these have easy harvesting technique and are capable of proliferating and differentiating into bone forming cells (for a review, see Kaveh et al., 2011). Another consideration is the source of such osteoprogenitor cells: autologous, allogenic or xenograft. Concerning the scaffold, this is usually three dimensional and highly porous with an interconnected pore network for cell growth and flow transport (nutrients, metabolic waste). Is biocompatible and bioresorbable. In addition, it should be capable of osteogenesis and osteoconduction (Moore et al., 2001). Its source can be synthetic such as poly lactic acid and poly glycolic acid bleds, bioactive glass

particles, hydroxyapatite/chitosan-gelatin networks, ceramic or glasses and bioactive glass or natural such as autograft or allograft, collagen, glycosamin glycans (Kaveh et al., 2009; Moore et al., 2001; Nuttelman et al., 2005; Vats et al., 2003). The scaffolds may be seeded with cells and cultured before surgery or the cells are seeded into the matrix and immediately implanted at the time of surgery (Kaveh et al., 2011).

Another interesting approach is the direct injection of cells (with or without signalling molecules) into the defect site. This may be a useful technique when the damage in the tissue is confined to a small area. Yu and co-workers have shown that the injection of polylactic acid-polyglycolic acid copolymer/collagen type I microspheres combined with bone MSCs improves intertrochanteric bone quality in osteoporotic female rats. In this work, rats were ovariectomized to establish the osteoporotic animal model. The animals that received the microspheres with the bone marrow MSCs had improved bone mineral density when compared to control animals that were treated with microspheres alone or PBS. This suggested that such approach may provide a promising minimally invasive surgical tool for enhancement of bone fracture healing or prevention of fracture occurrence (Yu et al., 2012).

The next chapters will describe the MSC's potential to differentiate into osteoblasts, the bone forming cells, since this is one of the main topics of this work. It relies on the possibility of using MSCs as osteoblast precursor cells in bone regeneration, namely in known bone disorders such as osteoporosis and rheumatoid arthritis.

1.1.3. The bone marrow microenvironment: a stem cell niche

As mentioned earlier, non-embryonic or adult stem cells have been identified in many organs and tissues. Some authors suggest that there is a single quiescent population of stem cells residing in a specialized niche of a given tissue (*e.g.*, bone marrow, brain, skin, skeletal muscle, adipose tissue) (Porada et al., 2006). The main properties of stem cells, namely the ability of differentiating into diverse specialized cells in the body and self-renewal, are believed to be maintained and

regulated by a specific microenvironment referred as *niche*. This local environment specifies the location of stem cells within tissues, as well as the cellular and molecular components that determine their activity (Rando, 2006). In other words, a stem cell niche is the microenvironment where the adult stem cell resides. It also includes the cells that surround them and the extracellular matrix, which are both believed to provide signals that keep the stem cell quiescent or, instead, modulate their activation. In this last case, stem cells undergo either symmetric division or asymmetric division, i.e. they give rise to daughter cells that are both stem or they produce one stem and other daughter cell that is already committed to differentiate. In fact, stem cell-function is regulated in a complex way, involving the cell-autonomous regulation to local environment stimuli of the stem-cell niche, the surroundings of the tissue, the so called systemic milieu of that organism, and the external environment (Figure 1) (Rando, 2006). The bone marrow is a key reservoir of stem cells. Here, cells interact with each other by releasing growth factors and cytokines, allowing cell survival and maintenance, as well as cell proliferation and/or differentiation (for a review, see Isern and Mendez-Ferrer, 2011).

We will focus our introduction in MSCs, since this work it is based on the ability of these cells to differentiate into osteoblasts, the bone forming cells.

MSCs seem to play a significant role in bone marrow microenvironment. One of their main roles is to create a tissue framework, assuring the mechanical support for the haematopoietic cell system. In addition, quiescent or stimulated cells can secrete a number of extracellular matrix proteins (*e.g*., fibronectin, laminin, collagen and proteoglycans) (Devine and Hoffman, 2000). Besides these proteins, MSCs (and to some extent, cells that derive from this lineage, like osteoblasts) also produce and secrete haematopoietic and non-haematopoietic growth factors, chemokines and cytokines, regulating haematopoiesis. This is depicted in Figure 2, showing a scheme of bone marrow as a stem cell niche. They secrete interleukins, namely IL-1a, IL-1b, IL-6, IL-7, IL-8, IL-11, IL-14, IL-15, macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor,

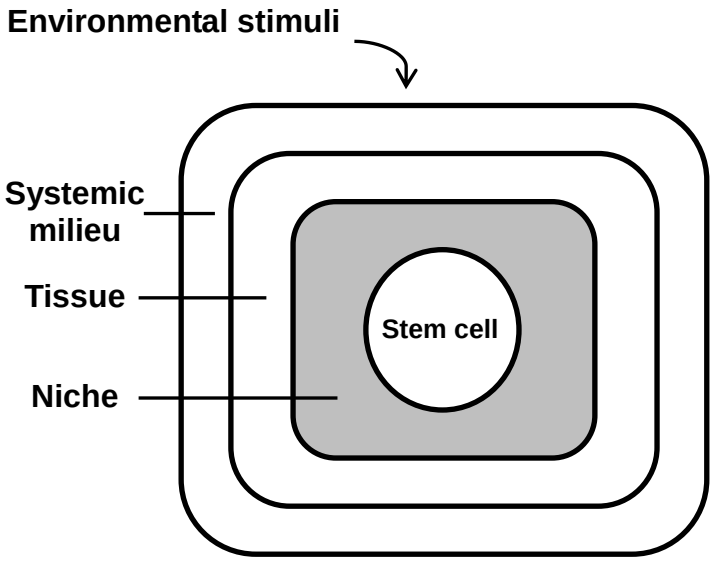

Figure 1. The stem cell niche. Stem cell niche is the microenvironment where adult stem cells reside. It includes the surroundings, cells and extracelullar matrix, which will provide the necessary signals for their quiescence or activation. External environmental influences also play a role, filter down through these levels to modulate stem cell fuction. See text for details (adapted from Rando, 2006).

leukaemia inhibitor factor, stem cell factor, foetal liver tyrosine kinase-3, thrombopoietin and hepatocyte growth factor (for a review, see Bobis et al., 2006).

Recent works have also suggested that co-transplantation of human MSCs and HSCs resulted in accelerated haematopoietic recovery in animal models and humans (Fibbe and Noort, 2003; Koc et al., 2000; Lane et al., 1999). It was found that transplanted MSCs in mice tibia integrate into the functional components of haematopoietic microenvironment and actively participate in the haematopoietic cell development, since 4-10 weeks later, MSCs differentiate into pericytes, myofibroblasts, osteocytes and endothelial cells, resulting in increased numbers of primitive human haematopoietic stem cells in the bone marrow microenvironment (Muguruma et al., 2006). In fact, many studies point towards an interaction between MSCs and HSCs. It was also found, for instance, that osteopontin production by MSCs significantly prevents HSCs proliferation (Denhardt and Guo, 1993). However, without mineralization, HSCs do not function properly (Adams et al., 2006). These findings support the concept that MSCs actively regulate the function of HSCs. The question remains whether HSCs influence the development and maintenance of the bone marrow niche. Recent reports suggest that HSCs

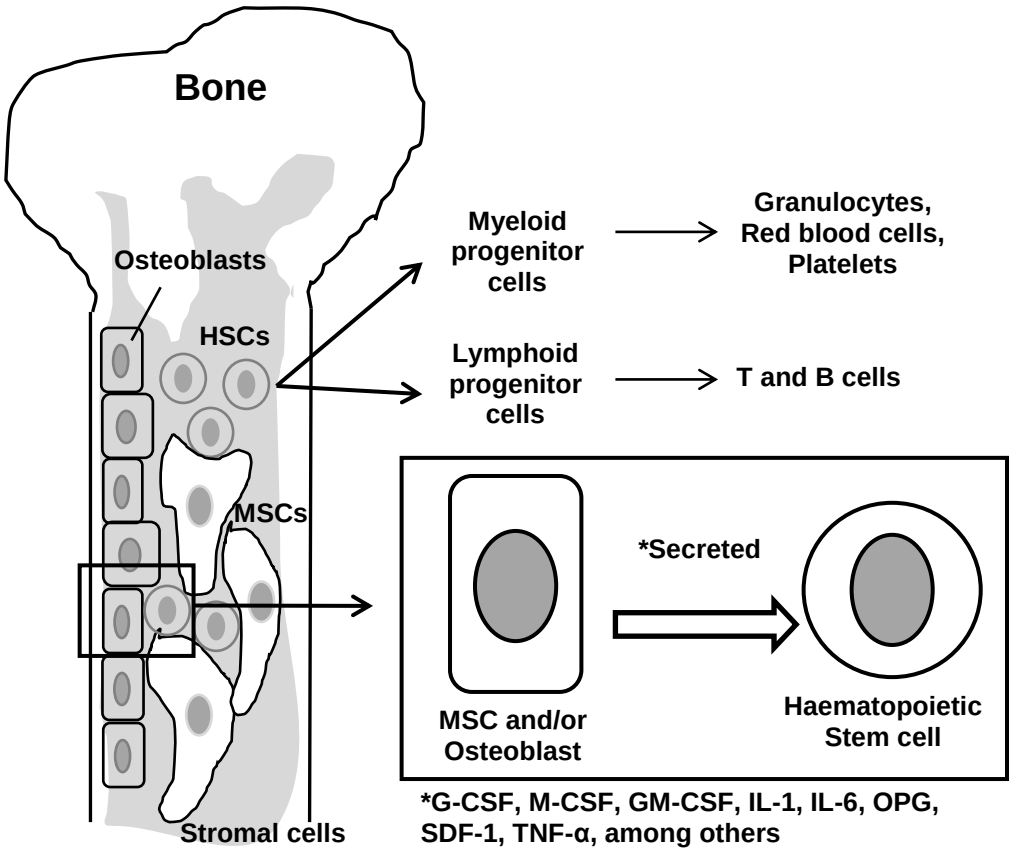

Figure 2. The bone marrow microenvironment. The stem cell niche is composed of several members of the bone marrow stem cell system. Recent studies demonstrate that endosteal osteoblasts and their precursors like mesenchymal stem cells (MSCs) play a critical role in the creation of a stem cell niche and thereby likely regulate stem cell maintenance, proliferation and maturation. In particular, osteoblasts and their precursors play a pivotal role in haematopoiesis by secreting chemokines and cytokines depicted in the figure. This will be important for the lineage commitment of haematopoietic stem cells (HSCs) (myeloid or lymphoid progenitor cells), leading to the differentiated cells displayed in the picture. G-CSF, granulocyte colony stimulating factor; M-CSF, macrophage colony stimulating factor; IL, interleukin; OPG, osteoprotegerin, SDF-1, stromal derived factor 1, TNF- α, tumor necrosis factor alpha (adapted from Taichman, 2005).

regulate bone formation through the production of BMP-2 and BMP-6 (Jung et al., 2008). Another particular example came from Liao and co-workers recent work. They have shown that *in vitro*, low dose of haematopoietic stem progenitor cells (HSPCs) co-cultured with MSCs in combination with dexamethasone accelerates the osteogenic progression of MSCs. They observed an earlier peak in alkaline phosphatase activity (an enzyme involved in the mineralization process) and

enhanced calcium deposition compared to MSC-cultures alone (Liao et al., 2011). The cellular mechanism is, however, still unknown.

Osteoblasts, which derive from the mesenchymal stem cell lineage, also play an important role in the bone marrow stem cell niche. Evidences came from different works in which several animal models were used, in which was studied a transcription factor, Runx-2, which belongs to the runt domain gene family. *RUNX-2*-deficient mice lack intramembranous and endochondral bone formation because osteoblasts don't mature (Komori et al., 1997). These animals are dwarfed, unable to breathe and die immediately after birth (Takarada et al., 2013). The lack of osteoblast maturation leads to a total lack of bone marrow through the entire skeleton. In the embryos, the animals develop excessive medullary haematopoiesis in the livers and spleens and large haematopoietic foci in the periportal area (Deguchi et al., 1999).These authors have shown that the migration of the haematopoietic precursors was apparently perturbed by a lack of bone marrow cavity in Runx-$2^{-/-}$ embryos (Deguchi et al., 1999). Other evidences reinforce the role of osteoblasts in the bone marrow microenvironment. These cells express, similarly to marrow endothelial cells, the stromal derived factor (SDF-1), which they constitutively release. When deleted in animal models, together with its receptor, CXCR4, marrow engraftment by haematopoietic cells is not observed (Aiuti et al., 1999; Peled et al., 1999). Some authors have also suggested that the production of SDF-1 by osteoblasts is an important mechanism for the selective attraction of circulating osteoclast precursors to bone and their migration within the marrow to appropriate perivascular stromal sites for RANKL differentiation (see below) into resorptive osteoclasts (Yu et al., 2003).

Other studies have also implicated osteoblasts as critical regulators of haematopoiesis. Zhang and co-workers have shown that bone morphogenic proteins (BMP), which may be produced and released by osteoblasts, regulate HSC development. They found that the conditional inactivation of the BMP receptor type IA (BMPRIA) in mutant mice leads to an increase in the number of HSCs when compared to the wild-type mice (Zhang et al., 2003). Calvi and co-workers also showed that parathyroid hormone (PTH), in osteoblasts

overexpressing the PTHR, enhances osteoblastic growth and simultaneously increases the number of HSCs in the marrow (Calvi et al., 2003). They also showed that the treatment of healthy animals with PTH similarly expands the HSC population in the marrows, since there is an expansion of osteoblast precursors. Again, administrating PTH to wild-type animals before HSC transplantation improves their survival. These results point to osteoblasts as stem cell-supportive cells in such niches.

Stem cells are able to maintain their undifferentiated state within the niche (Bobis et al., 2006). In the particular case of MSCs, some findings suggest that these cells decision to differentiate or to stay quiescent is regulated by Wnt family members. Its signalling is known to prevent differentiation process by inducing high levels of oct-3/4, rex-1 and the homeodomain transcription factor Nanog, which are known as gatekeepers for embryonic stem (ES) cell pluripotency (Sato et al., 2004). On the other hand, BMP-pathways play a role in the proliferation/differentiation process of stem cells, which raises the hypothesis that these factors are important for MSCs growth in their niche.

When needed, stem cells like MSCs may be recruited from the bone marrow to other tissues, circulating in blood, after particular stimuli (Bobis et al., 2006). They are kept in the cell niche since they express a number of adhesion molecules, which are also important for the niche function itself. These include N-cadherin/β-catenin, VCAM/integrin and osteopontin/β_1 integrin (for a revision, see Bobis et al., 2006). Osteoblasts themselves express many adhesion molecules that may also be used during cell-cell contact with HSCs. Some of these include VLA-4 ($\alpha_4\beta_1$) receptors (expressed on $CD34^+$ cells) and the vascular cell adhesion molecule-1 (VCAM-1) (expressed by bone marrow stromal cells) (Simmons et al., 1994). Other structures are involved, namely CD34, CD44, CD164, intracellular cell adhesion molecules (ICAM-1, ICAM-3), very late antigen-4 (VLA-4), among others (for a revision, see Verfaillie, 1998). Some cell-associated or matrix-bound cytokines, like IL-3, stem cell factor (SCF) and transforming growth factor α_1 (TGF-α_1) may themselves serve as adhesion molecules (Gordon, 1991; Hardy and Minguell, 1995; Toksoz et al., 1992). When migrating to other tissues, MSCs are

able to support the regeneration process. Such cases were documented in regenerating bone (where they differentiate into bone forming cells, the osteoblasts) (Benayahu et al., 1989), in cartilage repair (Caplan et al., 1997), muscle (De Bari et al., 2003), heart (Shake et al., 2002) and migration throughout the forebrain and cerebellum (where they differentiate into astrocytes) (Kopen et al., 1999). Homing of MSCs is possible thanks to the expression of chemokine receptors which will help them in trafficking to various tissues. An example is the CXCR4, the receptor for SDF-1, which is produced by stromal cells (Stoicov et al., 2013; Yang et al., 2013). Using such signalling mechanisms, MSCs detected in the bloodstream, are able to migrate and colonize various tissues (Gao et al., 2001).

1.2. Mesenchymal stem cells and osteogenic differentiation

1.2.1. Mesenchymal stem cells: *in vitro* induction of osteogenesis

When cells from the whole bone marrow are plated in plastic culture dishes with 10% of foetal calf serum supplemented medium, one finds that there is a population of haematopoietic non-adherent cells together with a rare population of plastic-adherent cells (in a proportion of 1:10000 nucleated cells in the bone marrow, varying from individual to individual) (Friedenstein et al., 1970). After medium change, only the adherent cells will remain. These will start to proliferate and can differentiate into mature cells of mesenchymal lineages such as osteoblasts (Friedenstein et al., 1970; Friedenstein et al., 1976). In fact, this ability to adhere to plastic is one of the criteria for the minimal identification of mesenchymal stem cells (MSCs) by the International Society for Cell Therapy in 2006 (Dominici et al., 2006). When assessed by the FACS analysis, these cells should also have the following cell phenotype: $CD73^+$, $CD90^+$, $CD105^+$, $CD45^-$, $HLA\text{-}DR^-$, $CD14^-$ or $CD11b^-$, $CD79a^-$ or $CD19^-$ (Dominici et al., 2006).

Osteoblasts are the bone forming cells and, as mentioned earlier, these derive from MSCs. *In vivo*, MSCs are believed to be in close relation with other cells, namely haematopoietic stem cells, forming the stem cell niche (see section 1.1.3). In order to promote the osteogenic differentiation of human MSCs *in vitro*, these

should be incubated with ascorbic acid, β-glycerophosphate and dexamethasone (in addition to foetal bovine serum) added to the culture medium (Jaiswal et al., 1997; Pittenger et al., 1999). Ascorbic acid is known to play a role in the collagen synthesis, since it is a cofactor for proline and lysine hydroxylases, both involved in the hydroxylation of collagen. Ascorbic acid seems to be essential for normal bone formation, since ascorbic acid was previously shown to increase alkaline phosphatase and osteocalcin mRNAs in osteoblast cultures (Franceschi and Iyer, 1992) and that this induction is blocked by inhibitors of collagen triple-helix formation. In addition, osteoblasts were shown to express a Na^+-dependent transporter specific for ascorbic acid which is essential for maintenance of intracellular ascorbate concentrations (Dixon et al., 1991; Franceschi et al., 1995) which, in turn, will influence the proliferation and alkaline phosphatase expression (Franceschi and Iyer, 1992; Franceschi et al., 1994). β-glycerophosphate is a substrate for alkaline phosphatase, producing an increase in phosphate content, which may be incorporated in the bone matrix (Bellows et al., 1991) together with Ca^{2+}. It functions as an organic phosphate donor and has been used in culture media for MSC differentiation to osteoblast-type cells (see *e.g.*, Jaiswal et al., 1997). Besides this, the free inorganic phosphate may induce increases in mRNA and protein expression of osteogenic markers like osteopontin and regulation of Runx-2 (Beck et al., 2000; Fujita et al., 2001). Dexamethasone is a synthetic corticosteroid. It mimics the actions of several glucocorticoids located naturally in the body (cortisol, estradiol, testosterone, vitamin D3, thyroxine and retinoic acid) (Kaveh et al., 2011). Glucocorticoid-activated genes work through gene activator proteins. These, when not associated to glucocorticoids, remain in the cytosol. When present, glucocorticoids bind to their glucocorticoid receptors (nuclear receptor subfamily 3, group C, member 1 - NR3C1) and to the activator proteins, migrate to the nucleus and bind to the regulatory region of each gene regulated in this manner. Dexamethasone supports osteogenic differentiation (Liu et al., 2002) by binding to some special regulatory proteins in the cell and then activating transcription of osteoblast-specific genes, like those coding for alkaline phosphatase, which is an enzyme required for matrix mineralization, converting

pyrophosphate (PPi, a known inhibitor of mineralization) into inorganic phosphate (Cheng et al., 1994). It is worth noting that dexamethasone *in vivo*, in long-term intake cases, results in osteoporosis and osteopenia (bone degeneration and loss) (Baylink, 1983). This apparent contradiction is based on the fact that chronic glucocorticoid intake leads to an inhibition of MSCs proliferation and may activate the action of osteoclasts. It was shown that $<10^{-8}$ M stimulated receptor activator of NF-κB ligand (RANKL)-induced osteoclast formation synergistically with transforming growth factor β (Takuma et al., 2003). So, regimens of dexamethasone may limit the amount of osteoprogenitors sources (Kaveh et al., 2011), resulting in an unbalanced bone formation versus bone resorption. Besides this, glucocorticoids are known to reduce Ca^{2+} absorption in the intestine, leading to an increase in circulating PTH, ultimately resulting in Ca^{2+} mobilization from bone (Fucik et al., 1975). This topic will be further discussed in section 1.3.2 which refers to bone metabolism description.

Dexamethasone treated cultures have increased ALP activity when compared to control cultures (Fernandes et al., 1997), consistent with other observations showing that long-term treatment of bone-derived cells with physiological concentrations of glucocorticoids induces their differentiation into cells with an osteoblast phenotype (Benayahu et al., 1989; Cheng et al., 1994). Fernandes and co-workers also revealed that dexamethasone treated cultures show randomly located multi-layered cell three-dimensional nodules, and that these appear to be important for *in vitro* mineralization of alveolar bone cells (Fernandes et al., 1997).

Besides the previous indicated factors, the standard procedure for *in vitro* culture of MSCs is based on supplementing cell culture media with foetal bovine serum (FBS). This contains growth factors and extracellular matrix molecules that enhance cell attachment to plastic surfaces, enhancing cell proliferation and differentiation. It provides several important biological molecules such as albumin, antichymotrypsin, apolipoproteins, biotin, and growth supporting factors, which are required for optimal growth of cells. In addition to the indicated factors, 10% of FBS was shown to be the indicated amount of serum for an osteoblastic-induction medium, although some variations may be found (Tateishi et al., 2008).

Although human bone cell cultures have been regarded as a useful tool to study bone control mechanisms, it should be emphasized that the number of passages in an *in vitro* study is an important factor to be considered. Several authors have demonstrated that serially passaged cells keep the proliferation rate constant, but without a similar ALP activity. This enzyme activity decreases with the number of passages and the ability to form mineralized areas also decreases on serial subcultures (Fernandes et al., 1997). In fact, the loss of the osteoblast phenotype on serial passage has been reported by other authors (Coelho et al., 2000). Other interesting findings revealed that serial passage of osteoblast-like cells, MC3T3-E1, alters their osteoblastic function and responsiveness to transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2). These authors showed that BMP-2 significantly enhances ALP activity and osteocalcin secretion in early passage cells while TGF-β1 has the opposite effect. Both BMP-2 and TGF-β1 effects significantly decrease on late passage cells. They also showed that ALP activity and osteocalcin secretion decreases after multiple cell passages (Chung et al., 1999). Taken all together, these evidences support the idea of a replicative senescence of *in vitro* cultures that may be a good starting point for cell ageing proposed *in vivo*. Besides this, data demonstrate that caution should be taken when considering serial passages of primary cultures, since cell phenotype and differentiation ability may be compromised.

1.2.2. Mesenchymal stem cells*:* osteogenesis regulation signals and gene expression

As mentioned earlier, mesenchymal stem cells (MSCs) are able to differentiate into multiple cell types, including osteoblasts. The latter are the bone forming cells, and together with osteoclasts (bone-destroying cells) and osteocytes (a type of cell that derives from the osteoblast lineage), compose the three major cells involved in bone remodelling. Bone formation, which is undertaken by osteoblasts (discussed further in section 1.3.2), involves a two-step process: first, mature osteoblasts synthesize and release type I collagen which constitutes 85-90% of the organic matrix and many other non-collagenous bone matrix proteins like

alkaline phosphatase (ALP), osteocalcin, osteonectin, osteopontin and bone sialoprotein. The organic matrix, known as osteoid, is subsequently mineralized by calcium and phosphate ions to produce calcified bone tissue, a mineral that is similar to hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (Orriss et al., 2010). This lineage commitment of multipotent MSCs is driven by the selective expression of one master transcriptional regulator, Runx-2 (runt-related transcription factor 2), which is necessary for the osteoblast lineage (reviewed in Jensen et al., 2010). This master regulator of osteoblastogenesis acts throughout the induction, proliferation and maturation of osteoblasts regulating the expression of many osteoblast genes, including those encoding osteocalcin, type I collagen and osteopontin (for a revision, see Franceschi and Xiao, 2003). Some of these genes are depicted in Table 1. This table summarizes some of the most important osteogenic markers, including the *RUNX-2* gene (also known as *CBFA1*). These are commonly chosen to evaluate, *in vitro*, the osteogenic differentiation of MSCs.

The Runx-2 activity and importance in osteoblastogenesis has been explored extensively, and studies in which mutations were induced in *RUNX-2* gene showed a number of skeletal abnormalities. One example is cleidocranial dysplasia syndrome, an autosomal-dominant skeletal dysplasia that is characterized by widely patent calvarial sutures, clavicular hypoplasia, supranumerary teeth and short stature. Besides this, homozygous mutation of *RUNX-2* in mice was shown to be lethal due to a complete lack of mineralized bone (Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997).

The signalling pathways regulating Runx-2 activity are still under extensive investigation, but there are several examples of such dynamic control. These include binding of extracellular matrix (ECM) to cell surface integrins, fibroblast growth factor 2 (FGF2), mechanical loading, parathyroid hormone (PTH) and bone morphogenic proteins (BMPs) (for a revision, see Franceschi and Xiao, 2003). Figure 3 summarizes these signalling pathways and how they interact to promote Runx-2 activity.

Table 1. Genetic and related protein markers of osteogenesis

Coding Gene	Protein name	Protein Function
c-fos	c-Fos	Belongs to the AP-1 class of transcription factors. Direct gene targets include those encoding osteocalcin, collagenase III, bone sialoprotein, alkaline phosphatase promoters (Jensen et al., 2010; Owen et al., 1990).
RUNX-2	Runx-2	Can directly stimulate transcription of osteoblast-related genes such as those encoding osteocalcin, type I collagen, osteopontin and collagenase III by binding to specific enhancer regions in DNA (Franceschi and Xiao, 2003).
ALPL	Alkaline phosphatase	Ubiquitous enzymes present in many organisms from bacteria to man. The most abundantly isoform expressed in bone is TNAP. Its major role is to hydrolyse PPi to maintain a proper concentration of this mineralization inhibitor ensuring normal bone mineralization (Yegutkin, 2008).
BGLAP	Osteocalcin	One of the most abundant matrix proteins in bones, synthetized by osteoblasts. It binds strongly to apatite and calcium, which is dependent on vitamin K (Gundberg et al., 2012).
SPP1	Osteopontin	One of the major phosphoproteins in bone. Osteoblast adhesion to bone matrix proteins such as osteopontin can modulate various aspects of cell behaviour, including growth, differentiation, and protein production (Liu et al., 2008a).
SP7	Osterix	Zinc finger-containing transcriptional activator that is distinctly expressed in all developing bones and is important for osteoblast differentiation, usually downstream of Runx-2. Osteocalcin, ALP and bone sialoprotein coding-genes are some of its targets (Sinha and Zhou, 2013).
COL1A1	Type I Collagen	Main constituent of the organic matrix synthesized by mature osteoblasts, which is released by exocytosis. Together with noncollagenous bone matrix proteins, constitutes the osteoid, which latter mineralizes with calcium and phosphate deposition to produce calcified bone tissue. It also induces the expression of several osteoblast-related genes through integrin mediated signal transduction (Mizuno and Kuboki, 2001).
IBSP	Integrin-binding sialoprotein	Noncollagenous glycoprotein in mineralized tissues such as bone. May be involved in cell attachment and signalling, hydroxyapatite nucleation, and binding of type I collagen (Malaval et al., 2008).
SPARC	Osteonectin	Glycoprotein belonging to a group of matrix associated factors that mediate cell-matrix interactions but do not serve primarily structural roles; has been shown to be a Ca^{2+}-binding glycoprotein. Seems to have a major role in bone remodelling or repair (Brekken and Sage, 2001).

These protein-coding genes are commonly used as markers of osteogenesis, *in vitro,* since there are extensive studies attesting their importance in this cell differentiation mechanism. See text and cited references for details.

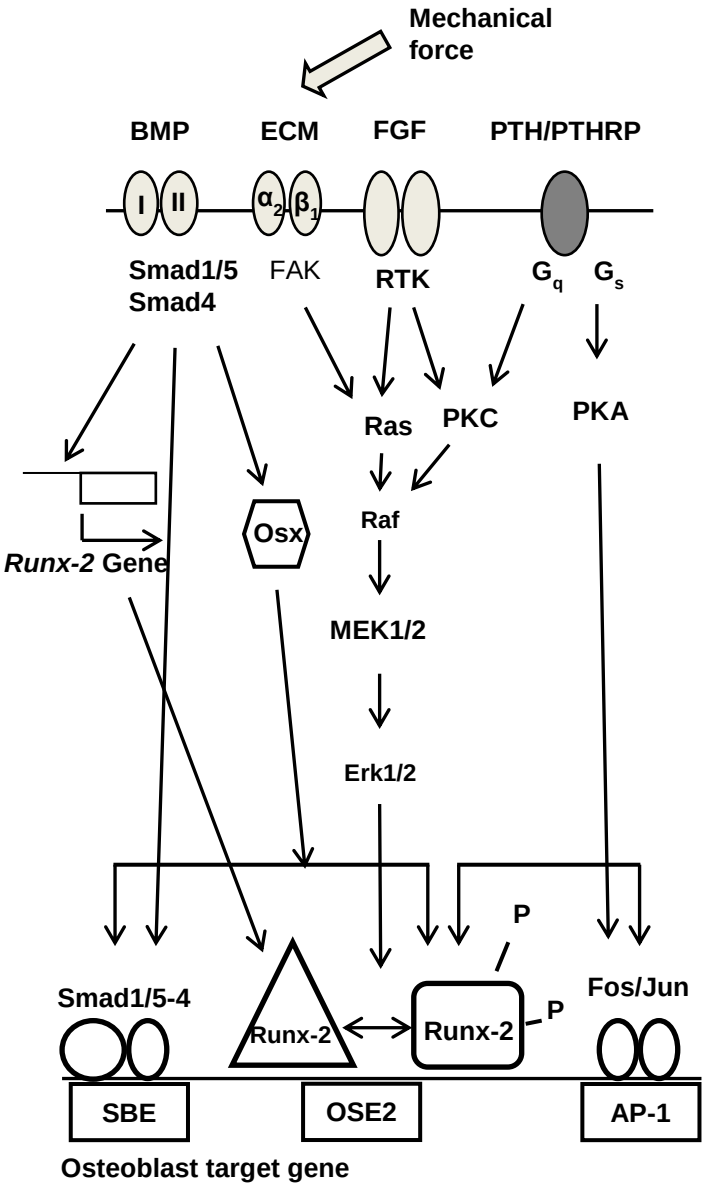

Figure 3. Signal transduction pathways modulating Runx-2 activity. This transcription factor is frequently described as the master regulator of osteoblastogenesis, since it acts throughout the induction, proliferation and maturation of osteoblasts and regulates expression of many osteoblast genes. See text for details. Based on Franceschi and Xiao, 2003.

ECM containing type I collagen is mandatory for osteoblasts to differentiate and express osteoblast-related genes such as those encoding osteocalcin, bone sialoprotein, alkaline phosphatase among others (Table 1) and, ultimately, to mineralize. Usually, the ECM signals through β_1 subunit-containing integrins (like $\alpha_2\beta_1$). This will promote differentiation of pre-osteoblast into mature osteoblasts. This sequence may be prevented using either blocking antibodies or peptides that

bind to the cell-binding domain of collagen, thus preventing ECM-dependent differentiation (Danen et al., 1998). Others have shown that ECM production by murine MC3T3-E1 cells increases significantly the transcription of osteocalcin coding gene, which is Runx-2 dependent (Xiao et al., 1997). Several authors suggested that the MEK/ERK branch of the mitogen activated protein kinase pathway, or MAPK, is a plausible linkage between integrin activation and subsequent stimulation of Runx-2-dependent transcription (reviewed in Franceschi and Xiao, 2003). A previous study showed that selective inhibition of ERK1/2 phosphorylation by MEK with U0126 prevented ECM-dependent induction of the osteocalcin coding gene. The authors showed that BMP action on osteogenesis was also prevented with the same blocker (Xiao et al., 2002). Other studies showed that transfecting cells with MEK1 results in Runx-2 phosphorylation, knowing that MEK1 is involved in the MAPK pathway (Xiao et al., 2000). In conclusion, evidences demonstrate that osteoblasts differentiation requires close contact with collagen-containing ECM, interacting with it through specific β_1-integrins. This interaction allows the activation of the MAPK pathway, which transduce signals to the nucleus. Subsequently, Runx-2 is phosphorylated and allows the stimulation of osteogenesis by increasing transcription of osteoblast marker genes such as those depicted in Figure 3.

As already mentioned, FGF2 is an important *in vivo* regulator of skeletal development and growth. Its administration can restore bone mass in the overiectomized female rat, which is a well-established model for postmenopausal bone loss (Liang et al., 1999). In transgenic mice, overexpression of FGF2 causes premature mineralization, achondroplasia and shortening of long bones. On the other hand, disruption of the *FGF-2* gene leads to decreased bone formation and mass (Coffin et al., 1995; Montero et al., 2000). Some other interesting findings revealed that activating mutations in FGFR1 up-regulate Runx-2, enhancing differentiation of calvarial osteoblasts (Zhou et al., 2000). FGF2 was also shown to promote osteocalcin gene expression in MC3T3-E1 pre-osteoblast cells (Boudreaux and Towler, 1996). The major route of FGF receptor signalling, as shown in Figure 3, involves the activation of MEK/ERK branch of the MAPK

pathway (Nugent and Iozzo, 2000). Xiao and co-workers also showed that FGF2 could rapidly induce ERK phosphorylation and increase osteocalcin mRNA, in both MC3T3-E1 and bone marrow stromal cells (Xiao et al., 2002).

Others revealed that FGF2 leads to osteocalcin production by promoting the transcription of its coding gene through an AP-1-like site that is immediately 5' to the *RUNX-2* binding site (Boudreaux and Towler, 1996) (Figure 3). AP-1 refers to a class of transcription factors that is composed of heterodimers of Fos-related factors (c-Fos, Fra1, Fra2 and FosB) and Jun proteins (c-Jun, JunB and JunD). These are highly expressed in proliferating osteoprogenitors.

The *c-fos* gene is usually evaluated to measure the extent of osteogenesis *in vitro* (Table 1). Accordingly, there are a number of direct gene targets of AP-1 in osteoblasts, namely those coding for osteocalcin, collagenase-3, bone sialoprotein and alkaline phosphatase (Table 1) (Owen et al., 1990).

Returning to FGF2, it was also shown that its response is synergistically stimulated by a PKA pathway activator (forskolin), which was also shown to increase the activity of AP-1 related nuclear factors (c-Fos and c-Jun). So, it seems that AP-1-like factors and Runx-2 share cooperative interactions in the promotion of osteogenesis (Franceschi and Xiao, 2003).

As depicted in Figure 3, mechanical stimulation is important in the regulation of bone homeostasis. Mechanical strained human osteoblast-like cells express increased levels of coding mRNA for osteopontin, osteocalcin and collagen I and III (Carvalho et al., 1998; Harter et al., 1995). The MAPK pathway seems to be largely involved in this process, and integrins seem to be associated to these mechanotransduction signals. These proteins connect the cytoskeleton to the extracellular proteins, mediating the transduction of mechanical stimuli into biochemical signals. One particular example of this observation are the experiments undertaken by Schmidt and co-workers, who showed that MAPK activation is related to $\alpha_2\beta_1$ integrins, when an osteoblastic cell line was submitted to mechanical stress, using a magnetic drag force device (Schmidt et al., 1998). Ziros and co-workers also showed that osteoblast differentiation is promoted when mechanical stretching is applied, promoting the binding of Runx-2 to OSE2 DNA in

gel retardation mobility shift assays (depicted in Figure 3), also promoting ERK1/2 phosphorylation (Ziros et al., 2002). Similar results were also found in bone marrow stromal cells from three-month-old Sprague Dawley rats, in which mechanical force promoted osteogenic differentiation (Wang et al., 2002).

Another important recognized modulators of Runx-2 activation, and so of osteogenesis, are the parathyroid hormone (PTH) and bone morphogenic proteins (BMPs). PTH is an important regulator of calcium homeostasis (further described in section 1.3.2 below). This hormone has both anabolic and catabolic effects on osteoblasts activity. This hormone functions by binding to the G-protein coupled PTH-1 receptor, or PTH1R, activating two possible pathways: protein kinase A (PKA), with previous activation of $G\alpha_s$ protein and adenylate cyclase resulting in the production of cAMP; and protein kinase C, with previous activation of the $G\alpha_q$ protein, with subsequent activation of phospholipase Cβ which leads to the formation of both DAG (which activates some isoforms of PKC) and IP3 (1,4,5-inositol trisphosphate), which recruits intracellular calcium from the endoplasmic reticulum. Both PKA and PKC pathways can regulate transcription factors such as cAMP response element binding proteins (CREBs), AP-1 family members, as well as Runx-2 (for a review see Karaplis and Goltzman, 2000). In fact, PTH seems to stimulate the collagenase 3 promoter by a PKA-dependent pathway that phosphorylates Runx-2 and up-regulates c-Fos/c-Jun (for a review see Franceschi and Xiao, 2003) (Figure 3).

BMPs are one of the best studied inducers of osteoblast and chondrocyte differentiation. These proteins bind to type I and II BMP receptors. The signalling transduction is undertaken by Smad proteins (Smad 1, 5 and 8, or R-Smads and Smad 4) (Baker and Harland, 1997). These proteins interact with each other and with enhancer sequences of target genes (also known by SBEs or Smad binding elements). This leads to the up-regulation of transcription factors such as Runx-2 and the zinc finger factor named Osterix (Table 1 and Figure 3) (Watanabe and Whitman, 1999). In fact, it has been reported that BMP treatment induces osteoblast-specific gene expression in MC3T3-E1 cells (Xiao et al., 2002) and that BMP-2 enhances ALP activity and osteocalcin production in human bone marrow-

derived primary osteoblasts (Jorgensen et al., 2004). In fact, several studies suggest a cooperative interaction between BMP-2 and Runx-2 signals. By using a C3H10T1/2 mesenchymal cell line, Yang and co-workers showed that there is a synergistic effect of BMP-2 and Runx-2 on osteogenic differentiation. These authors used adenoviruses to transfect cells with both Runx-2 and BMP-2. They saw a small increase in ALP activity and osteocalcin mRNA when cells were transfected with Runx-2 virus or BMP-2 virus, but when both were present, a significant increase (by 10-fold) in osteocalcin mRNA was observed (Yang et al., 2003).

Other important factors influence osteogenesis, namely the Wnt signalling (previously mentioned) and the Notch signalling pathways. In particular, the latter has dimorphic effects on mesenchymal progenitor cell lineage, since it supresses its differentiation into osteoblasts while maintaining the mesenchymal progenitor cell phenotype (Hilton et al., 2008). It can command early osteoblastic proliferation, while inhibiting osteoblast differentiation and maturation.

Recently, new insights came out concerning the role of micro RNAs (miRs) in the regulation of osteogenesis. These are short noncoding RNAs with 18-25 nucleotides, which are able to regulate gene expression through binding to the 3'-UTR of mRNAs for specific target genes. They are able to inhibit gene expression by promoting degradation of the target mRNAs or by inhibiting its translation (Erson and Petty, 2008). Some miRs function as inhibitors of osteoblastogenesis, while others promote it. For example, Li and co-workers found that a set of micro RNAs are reduced by BMP-2 stimulation of C2C12 mesenchymal cells. These twenty two identified micro RNAs were predicted to inhibit a range of pro-osteogenic factors (Li et al., 2008). Another example is the involvement of two specific miRs named miR-29a and miR-29c which are expressed in response to canonical Wnt signalling and inhibit expression of the extracellular matrix protein osteonectin (Kapinas et al., 2009). On the other hand, miR-141 and miR-200a were shown to be involved in BMP-2-induced mouse pre-osteoblast differentiation by interacting with Dlx5 coding gene. This gene encodes to Dlx5 protein that plays a role in bone development and fracture healing. Mutation in this gene may be

associated with split-hand/split-foot malformation (Itoh et al., 2009). This approach may be of clinical relevance, since micro RNAs have uncovered new mechanisms for regulation of skeletal physiology.

Many other factors induce osteogenesis which is known to be important in both *in vitro* and *in vivo* bone remodelling. Some of these will be further discussed in section 1.3.2, in the context of bone metabolism. Purinergic signalling, one of the main topics of this work is an important aspect when considering osteogenic modulation. This topic will be extensively discussed in section 1.4 below.

1.2.3. Mesenchymal stem cells: loss of function and stem cell ageing

Aging is characterized by a decline in organ/tissue maintenance and repair. Since adult stem cells are responsible, in part, for regeneration, it is conceivable that ageing is partly due to a decline of stem cell function. Some possible explanations may lie at the molecular level. Ageing is associated to an accumulation of damage affecting DNA, proteins, membranes and organelles (Ju and Rudoplh, 2008). One possible explanation for DNA damage is telomere shortening, which represents a cell intrinsic mechanism of ageing. DNA damage, in turn, leads to cell cycle arrest or cell death.

Telomere is a nucleoprotein complex that protects each end of a chromosome. It consists of non-coding TTAGGG double strand repeats, a 3' single strand overhang and associated telomere binding proteins (Blackburn, 1991). Telomere prevents DNA damage responses at chromosome ends, preventing fusion, degradation and instability (Blackburn, 2001). In fact, in humans, it is now clear that telomeres shorten with each round of cell division (Harley et al., 1990). The loss of sequence is due to the so-called end-replication problem of DNA polymerase, to the postreplication processing of telomere ends and to other factors such as oxygen species (Sfeir et al., 2005; Shay and Wright, 2000; von Zglinicki, 2002). At this stage, the cell requires an enzyme to counterbalance this feature, the telomerase. This ribonucleoprotein elongates telomeres by synthesizing six-nucleotide repeats (Tumpel and Rudolph, 2012). This enzyme consists of two important components: the telomerase RNA component (TERC),

which serves as a template for synthesis of telomeres, and the telomerase reverse transcriptase (TERT), which is the catalytic subunit of this enzyme (Ju and Rudoplh, 2008). Telomerase activity, in human cells, is repressed mostly in somatic tissues due to the suppression of TERT expression and most differentiated organ cells show a complete absence of TERT expression, with no telomerase activity (Wright et al., 1996). In contrast, stem cells are one of the few cells in adult humans that express telomerase (Chiu et al., 1996). Nevertheless, with aging, telomerase activity is insufficient to maintain stable telomeres in stem cell compartments, leading to DNA damage. One such example is telomere shortening in haematopoietic stem cells (HSCs) during ageing, limiting their self-renewal (Morrison et al., 1996). Some authors showed that proliferative stress in mouse experiments accelerates telomere shortening in HSCs in serial transplantation experiments (Allsopp et al., 2001) and that in TERC knockout HSCs telomerase shortening is increased when compared to murine HSCs (Allsopp et al., 2003).

So far, we have described the ability of mesenchymal stem cells to differentiate into multiple cell lineages, including osteoblasts. However, growing evidence has shown a decline of this ability with age, which is thought to influence therapeutic efficacy (Raggi and Berardi, 2012). In fact, telomere shortening due to telomerase deficiency leads to accelerated senescence of human skeletal mesenchymal stem cells *in vitro*, whereas overexpression of TERT leads to telomere elongation, ultimately resulting in enhanced bone formation (Simonsen et al., 2002; Stenderup et al., 2003). Such data strongly suggests that telomere shortening and telomerase activity are important factors in bone formation (Saeed et al., 2011). In fact, recently, Saeed and co-workers showed that telomerase-deficient mice exhibit bone loss. They showed that TERC deficient mice exhibit accelerated age-related bone loss (3-12 months) revealed by X-ray (Saeed et al., 2011). MSCs and osteoprogenitors isolated from TERC deficient mice exhibit a reduced *in vitro* proliferation capability and impaired osteogenic differentiation capacity, with lower levels of Runx-2 and ALP mRNAs, as well as a reduced mineralization at a later stage of the cultures, when compared to MSCs isolated from wild-type animals

(Saeed et al., 2011). Another interesting finding showed that hormones seem to have an effect on telomere length regulation. In fact, *in vivo* studies revealed that oestrogen and telomere shortening are linked. In that study, the authors reported that oestrogen deficiency in mice (targeted disruption of the aromatase gene) results in a significant inhibition of telomerase maintenance of telomeres in mouse ovaries in a tissue-specific manner. They also showed that oestrogen replacement therapy leads to increases in TERT coding gene expression, telomerase activity, telomere length and ovarian tissue growth, thereby reinstating ovary development to normal (Bayne et al., 2011). Since oestrogens are important regulators of bone remodelling, their lack during menopause may compromise MSCs capability to differentiate into osteoblasts due to telomerase deficiency. In fact, other authors showed that TERC mutant mice have a low bone mass phenotype, and that age-related osteoporosis is the result of impaired osteoblast differentiation in the context of intact osteoclast differentiation (Pignolo et al., 2008).

Some reports point to age-related changes in the number of bone marrow stem cells (Sudo et al., 2000). This may contribute to impaired bone formation, since less available osteoprogenitors (MSCs) will limit the number of mature osteoblasts needed for new bone formation (Kassem and Marie, 2011). This observation was also reported for the haematopoietic system (Sudo et al., 2000), and as mentioned above, haematopoietic stem cells are important for the bone marrow niche.

Besides this, some authors suggested that the life-span of osteoblasts decreases with age. They have reported an age-related increase in cell apoptosis, which may be related to an enhanced oxidative stress with age (Almeida et al., 2007). Zhou and co-workers showed that there is an increase in apoptotic cells in MSC cultures (Zhou et al., 2008), but further studies are needed to explain this phenomenon. Regarding oxidative stress, it has been reported that both *in vitro* and *in vivo* free radicals lead to impaired osteoblastic function and bone formation during aging (Manolagas, 2010). It has been shown in both aging female and male C57BL/6 mice an increase in reactive oxygen species (ROS) levels, decreased glutathione reductase activity and a corresponding increase in the phosphorylation of p53 and $p66^{shc}$, two key components of a signalling cascade that are activated

by reactive oxygen species and that influence apoptosis and lifespan (Almeida et al., 2007). Authors showed that these results are reverted upon treatment with oestrogens or androgens *in vivo* as well as *in vitro*. Age-related decrease in osteoblast function may also be related to the observed decrease in osteoblastic response to calciotropic hormones and growth factors. It is largely known that osteoblast cell proliferation and function depend on adequate responses to such factors in order to control bone formation. For instance, in mice, it has been reported a decrease in insulin-like growth factor (IGF-I) effects due to aging (Cao et al., 2007). Osteoblastic cells from aged human donors exhibit decreased proliferative responses to growth hormone and platelet-derived growth factor compared with cells from younger donors (Pfeilschifter et al., 1993). Other studies showed that osteoblasts from younger donors exhibit a better response to oestradiol and IGF-I when compared to older donors (Ankrom et al., 1998; D'Avis et al., 1997).

In conclusion, expanding data in literature indicates that age-related impaired bone formation is the main pathogenic mechanism mediating bone-loss. There are still a limited number of anabolic therapeutic approaches for treating bone disorders such as osteoporosis (further discussed below in section 1.3.3), but targeting senescence-related osteoblastic dysfunction may create novel anti-aging therapeutic possibilities, which may prove to be useful in promoting bone formation and reducing bone loss with age.

1.3. Bone: a dynamic and specialized tissue

1.3.1. Structure of bone: an introduction

Previously in this work, we have mentioned that the bone marrow is a key reservoir of stem cells which is housed in the medullary cavity of bone. In this topic, we will briefly describe bone as a dynamic tissue that is under continuous remodelling, with other functions besides a stem cell reservoir.

Bone, as a specialized connective tissue, contains inorganic mineral salts deposited within an organic collagen matrix and cells, namely osteoblasts,

osteoclasts and osteocytes. In order to repair, to grow and adapt, the bone needs to undergocontinuous remodelling. Throughout life, many abnormalities in such balanced process may result in a huge variety of bone disorders and skeletal abnormalities (further discussed below) (Orriss et al., 2010).

Together with cartilage, bone makes the skeletal system, holding three important functions: mechanical, protective and metabolic. Mechanical, since it bears a supportive role and is a site of muscle attachment; protective, since it shields vital organs and bone marrow; metabolic, since it is a reservoir of ions for the entire organism, in particular calcium and phosphate (Adler, 2000). Its main constituents are the cells and the extracellular matrix. The extracellular matrix is composed of collagen fibres and noncollagenous proteins (see below). The matrix of bone has the unique ability to calcify (Clarke, 2008).

Non-collagenous proteins together with collagen and water, are the main constituents of bone matrix. There are two main forms of extracellular matrix (ECM): the osteoid and mineralized matrix. Osteoid is an immature form of matrix excreted by osteoblasts and it's found in areas of new bone formation. It is subsequently mineralised by calcium and phosphate ions to produce calcified bone tissue; the mineral approximates to hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (Orriss et al., 2010).This mature form of matrix is largely mineral, being calcium and phosphate the main constituents. The turnover of bone minerals is highly regulated in a process known as bone remodelling. The extracellular matrix gives the bone its mechanical properties but is also important for regulation and formation of new bone (Baron, 1993).

There are four major cell types within bone tissue itself: osteoclasts, osteoblasts, osteocytes, and bone lining cells. Within the cavities of the bone, there is also bone marrow, which has numerous cell types, including the progenitor cells for the haematopoietic cell lineages (Baron, 1993). The osteoblast is the cell responsible for construction of new osteoid (which eventually becomes ECM). It is also the osteocyte and the bone lining cell precursor, also involved in osteoclast regulation (Boyce, 2013). The osteoblast is derived from the mesenchymal marrow stromal cells as mentioned above. TGF-β, BMPs, PTH, and

vitamin D are all important in stimulating MSCs to become osteoblasts (Deng et al., 2008). MSCs can be found in both the bone marrow and the inner layer of the periosteum. Osteoblasts can be incorporated into the osteoid and become osteocytes, line the bone and become bone lining cells, or undergo apoptosis. They are stimulated by PTH, 1,25-hydroxyvitamin-D, and insulin-like growth factor (IGF)-I (Datta et al., 2008).

The mature osteoblast is designed for protein synthesis: it has a large and efficient rough endoplasmic reticulum, Golgi apparatus, and secretory vesicles. Osteoblasts are polarized, with their synthetic functions at one end (near the cellular attachment areas) and their regulatory functions and nucleus at the other (Deng et al., 2008).

Bone lining cells, which derive from osteoblasts, no longer play a role in synthesis. These flat thin cells have little metabolic activity, covering non-metabolically active areas of the bone. However these play an important role in bone resorption: it is the peelback of the lining cells that stimulates and allows the attachment of osteoclasts to bone (Everts et al., 2002). Osteocytes are derived from the osteoblasts. During the formation of new bone, these become embedded in the bony matrix and differentiate into osteocytes. These cells form a connected cellular network that, along with the nerve fibres in bone, has a role in the response to mechanical loading (Kringelbach et al., 2014), since they sense mechanical strain and cracking, and respond by triggering bone remodelling. To balance this effect they can secrete sclerostin, which reduces bone formation (Kogawa et al., 2013). It survives in single cell-sized hole in the bone known as a lacuna. It still plays a vital role in bone homeostasis, although different from the osteoblast. 90% of all bone cells are osteocytes, and they can survive for decades. These are interconnected to one another through long cellular projections in tunnels known as canaliculi. Additionally, these tunnels serve as the source of nutrients and disposal of waste for these cells (Milovanovic et al., 2013).

The osteoclast derives from the haematopoietic macrophage lineage. The osteoclast is a multinucleated giant cell that is responsible for bone resorption, usually found in contact with a calcified bone surface and within a lacuna

(Howship's lacunae). The contact zone with the bone is characterized by the presence of a ruffled border and the attachment proteins that allow it to seal itself to the bone surface and pump carbonic acid (Baron, 1993). In addition to this acidic environment, the osteoclast synthesizes enzymes that degrade extracellular matrix proteins. Its function is intimately tied to the osteoblast cells (see below). This paired activity of bone-building and bone-absorbing cells is known as coupling and is crucial to the regulation of bone and calcium in the body (Ikeda and Takeshita, 2014)

1.3.2. Bone remodelling and metabolism

As mentioned earlier in this thesis, bone has multiple functions in vertebrates, including protection of vital organs and haematopoietic marrow, structural support for muscles, and storage and release of vital ions, such as calcium, and of growth factors which are stored in the matrix. Bone remodelling is the process in which bone, in the adult skeleton, is renewed continuously in response to a variety of stimuli. The osteoclasts are involved in the removal of trenches or tunnels of bone from the surfaces of trabecular and cortical bone, respectively (Boyce, 2013).

Osteoblasts subsequently fill in these trenches by laying down new bone matrix in them. Bone formation matches resorption during normal bone remodelling (Boyce and Xing, 2008). Bone remodelling involves: (1) the activity of osteoblasts and osteoclasts; (2) the actions of a variety of cytokines; (3) the turnover of bone minerals, particularly calcium and phosphate (although other minerals, such as magnesium, may also be important); (3) the actions of several hormones, such as parathyroid hormone (PTH), the vitamin D family, oestrogens, growth hormone, steroids, calcitonin and various cytokines (such as interleukins). Many other factors affect bone remodelling, such as exercise, diet and drugs (Feng and McDonald, 2011).

Bone remodelling starts with recruitment of osteoclast precursors and the subsequent differentiation of these to mature multinucleated osteoclasts induced by cytokines. Osteoclast precursors (OCPs) are attracted from the bone marrow (where they are held by the stroma-derived factor-1, SDF-1) to the bloodstream by

chemokines and circulate there until they are attracted back into bones by a variety of factors released at sites undergoing resorption, called bone remodelling units (BRUs), differentiating into osteoclasts (Boyce et al., 2012). These factors include the macrophage-colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (Boyce et al., 2012). Osteoclasts adhere to an area of trabecular bone, developing a ruffled border at the attachment site. H^+ ions are pumped through the ruffled border and, along with Cl^-, form HCl, which demineralizes bone, and cathepsin K is secreted to degrade the matrix (Boyce, 2013). This process gradually releases IGF-I and TGF-β, which were trapped in the osteoid. These cytokines recruit and activate osteoblasts cells, which invade the site, synthesising and secreting the organic matrix of bone, the osteoid, and secreting IGF-I and TGF-β (see *e.g*. Chernausek et al., 2007, and Janssens et al., 2005) Some osteoblasts become embedded in the osteoid, differentiating into osteocytes; others interact with and activate osteoclast precursors, the process known as coupling (see above).

Besides IGF-I and TGF-β, other cytokines belonging to the members of the TGF-β family, such as the bone morphogenic proteins (BMPs), a range of interleukins, various hormones and members of the tumour necrosis factor (TNF) family are involved in bone remodelling. RANK, receptor activator of nuclear factor kappa B (NFκB), is a central receptor involved in osteoclast differentiation, being NFκB the principal transcription factor involved. It is activated by RANKL, which exists as a homotrimeric protein and is typically membrane-bound on osteoblastic and activated T cells or is secreted by some cells, such as activated T cells (Kearns et al., 2008). Like other TNF receptor family members, RANK lacks intrinsic kinase activity to phosphorylate and activate downstream signalling molecules; it recruits TNF receptor-activating factors (TRAFs), particularly TRAFs 1, 2, 3, 5, and 6, which are adapter proteins that recruit protein kinases (Boyce, 2013). Briefly, this process allows the activation of NIK (NF-κB-inducing kinase), and signalling through this kinase and TRAF6 results in the activation of NF-κB and subsequent increased expression of *NFATc1* (which has been called the

master regulator of osteoclastogenesis) to induce further osteoclast progenitor differentiation (Boyce, 2013).

Osteoblasts also produce osteoprotegerin (OPG), a decoy ligand that inhibits osteoclast differentiation by blocking the RANK receptor. During coupling, OPG can bind to RANKL and inhibit RANKL's binding to the functional receptor, RANK, on the osteoclast precursor cell. RANKL/OPG ratio is critical in the formation and activity of osteoclasts and, thus, the optimal functioning of the RANK, RANKL and OPG system is crucial for bone remodelling (Boyce and Xing, 2008; Wright et al., 2009). Oestrogens induce osteoprotegerin synthesis, which may be one mechanism by which exogenous oestrogens inhibit osteoclast function and bone resorption in postmenopausal women (see section 1.2.3).

As mentioned above, bone remodelling involves daily turnover of bone minerals, being calcium and phosphate the most important. Regarding calcium, 99% of this mineral is in bone and teeth. Normal serum calcium varies from 2.1 to 2.6 mM. Three different components may be found: ionized (approximately 50%), protein-bound (nearly 40%, mainly albumin) and complexed to anions such as phosphate and citrate (approximately 10%). It is the ionized form (approximately 1.2 mM) that exerts the biological effects, and so it is tightly regulated. Hormones regulate plasma Ca^{2+} by controlling Ca^{2+} absorption from the intestine and its excretion by the kidney. These hormones may also recruit Ca^{2+} from the skeletal reservoir (Rang et al., 2011).

Ca^{2+} enters the body through the intestine by facilitated diffusion (majority of total calcium uptake) throughout the small intestine and by active transport in the proximal duodenum (regulated by vitamin D). Glucocorticoids or phenytoin depress intestinal calcium transport. There is a calcium loss due to mucosal and biliary secretions and sloughing of intestinal cells. Calcium excretion in the kidney is also highly regulated. Around 9 g of Ca^{2+} is filtered in the glomeruli, of which >98% is reabsorbed in the tubules. The efficiency of tubular reabsorption is regulated by PTH and influenced by filtered Na^{+}, the presence of non–reabsorbed anions, and diuretics. Regarding phosphate, approximately 80% of its total is found in bone and around 15% in soft tissues. It is present in collagen, bone,

plasma, extracellular fluid, cell membrane phospholipids and in the intracellular fluid. Phosphorus exists in both organic and inorganic forms in the body. The former includes phospholipids and organic esters. In the extracellular fluid, phosphorus exists as inorganic phosphate (Pi) in the form of NaH_2PO_4 and Na_2HPO_4. Pi interferes with tissue concentrations of Ca^{2+} and plays a major role in renal acid excretion. In bone, phosphate is complexed with calcium as hydroxyapatite and calcium phosphate (see above). Phosphate uptake in the intestine is passive, although an active component may be stimulated by several factors, including vitamin D. Its excretion in the kidney is highly regulated. More than 90% of plasma phosphate is filtered at the glomeruli, and more than 80% of which is reabsorbed in the tubules. Renal phosphate reabsorption is regulated by many factors, including PTH and dietary phosphate (for a review, see Moe, 2008). The most important hormones that regulate Ca^{2+} and phosphate concentrations are PTH and 1,25-dihydroxyvitamin D (or calcitriol), which regulate mineral homeostasis by effects on the kidney, intestine, and bone. Besides these, other hormones are involved in bone metabolism and remodelling, such as oestrogens, calcitonin and thyroid hormone (for a review, see Raisz, 1999).

PTH, parathyroid hormone, consists of a single polypeptide chain of 84 amino acids. It acts on PTH receptors in various tissues (like bone, kidney and gastrointestinal tract) and its role is to maintain a constant concentration of Ca^{2+} in the extracellular fluid. Parathyroid hormone is synthesised in the cells of the parathyroid glands, stored in vesicles, and its release is controlled by the concentration of ionised calcium in the plasma. Calcium acts via the calcium-sensing receptor (CaSR), a GPCR that couples with G_q-PLC and G_i. Occupancy of the CaSR by Ca^{2+} inhibits PTH secretion (in a case of hypercalcaemia); on the other hand, hypocalcemia stimulates PTH secretion (Bisello et al., 2004; Deal, 2009).

In tissues, PTH binds to the PTH type 1 receptor, a class II G protein-coupled receptor that is abundant in kidney and bone (in osteoblasts and osteocytes), and triggers classic G-protein signalling pathways, including G_s-linked cAMP production and $G_{q/11}$-dependent activation of phospholipase C (PLC) β and

subsequently calcium transients and protein kinase C (PKC) activation (Sneddon et al., 2004). In the kidney, PTH enhances the efficiency of Ca^{2+} reabsorption (primarily in the distal nephron through the stimulation of the epithelial Ca^{2+} channel TRPV5), inhibits tubular reabsorption of phosphate, and stimulates conversion of 25-OH-ergocalciferol (25-OHD) to calcitriol, since it stimulates 1α-hydroxylase, enhancing the synthesis and release of calcitriol. As a result, Ca^{2+} plasma concentration increases, whereas phosphate is excreted and its plasma concentration falls. Calcitriol, in turn, will interact with vitamin D receptors (VDRs) in the intestine to increase the efficiency of calcium absorption, and so promotes the increase of plasma Ca^{2+} concentration (see *e.g.* Li et al., 1998).

The skeletal actions of PTH are complex, since this hormone can stimulate both osteoblast-dependent bone formation and osteoclast-mediated bone resorption; PTH increases bone resorption and thereby increases Ca^{2+} delivery to the extracellular fluid. The major effects of PTH on differentiated osteoblasts are dependent upon binding to the transmembrane PTH/PTHrP receptor type 1 (PTHR1) and activation of transcription factors such as the activator protein-1 family, Runx-2 and cAMP response element binding protein (CREB); much of the regulation of these factors by PTH is protein kinase A (PKA)-dependent, (Swarthout et al., 2002), promoting osteoblastogenesis. On other hand, several studies suggest that continuous (but not intermittent) PTH can result in an increase in receptor activator of nuclear factor-kB ligand (RANKL) expression and consequent osteoclastogenesis in culture, with an associated inhibitory effect on osteoprotegerin expression (Locklin et al., 2003; Ma et al., 2001).

Vitamin D (calciferol) consists of a group of lipophilic pre-hormones that are converted in the body into a number of biologically active metabolites that function as hormones (Reichel et al., 1989). Their main action is mediated by nuclear receptors of the steroid receptor superfamily (VDR), and consists in the keeping of plasma Ca^{2+} by increasing its absorption in the intestine, mobilising Ca^{2+} from bone and decreasing its renal excretion circulating in the blood. It also regulates the activities of various cell types, namely osteoblasts (Anderson et al., 2013). In humans, there are two sources of vitamin D, dietary ergocalciferol (D_2), derived

from ergosterol in plants, and cholecalciferol (D_3) generated in the skin from 7-dehydrocholesterol by the action of ultraviolet irradiation (Norman, 2008). In this particular mechanism, exposure to ultraviolet light in sunlight converts 7-dehydrocholesterol to cholecalciferol (vitamin D3) in the skin. In liver, hydroxylation occurs to form 25-OH-cholecalciferol or 25-OH-ergocalciferol (25-OHD). After production in the liver, 25-OHD enters the circulation, bound to vitamin D-binding protein and its final activation occurs predominantly in the kidney, where 1α-hydroxylase in the proximal tubule converts 25-OHD to calcitriol. In the kidney, calcitriol stimulates Ca^{2+} reabsorption in the distal tubule. In the proximal duodenum, calcitriol stimulates TRPV6 Ca^{2+} channels and also induces the synthesis of calbindin D9K, calbindin D28K, and the serosal membrane Ca^{2+}-ATPase, promoting calcium uptake. In the absence of calcitriol, passive diffusion via the paracellular pathway is the mechanism involved (Morris and Anderson, 2010).

Regarding bone, vitamin D has a direct effect on bone cells. Osteoblasts, osteoclasts and osteocytes are capable of converting 25-OHD into calcitriol (Morris and Anderson, 2010). Human and rodent osteoblasts express the CYP27B1 enzyme (or 1α-hydroxylase) which is essential to convert 25-OHD to calcitriol and to increase expression of key genes associated with maturation and mineralization, the calcitriol responsive genes osteocalcin and osteopontin (Atkins et al., 2007). In the transgenic mouse model of osteoblast and osteocyte VDR overexpression, an increased bone volume and strength due to increased mineral apposition as well as decreased osteoclast formation was shown, proving the direct action of vitamin D on osteoblasts. However, on bone absorptive cells, the osteoclasts, it was found that these express cytoplasmic CYP27B1 and nuclear VDR proteins. In pre-osteoclasts, such as in human peripheral blood mononuclear cell preparations, CYP27B1 expression was necessary for 25D to optimise osteoclastogenesis in the presence of RANKL and M-CSF in vitro (Kogawa et al., 2010). So, vitamin D action in bone is complex and goes beyond the Ca^{2+} increase in the plasma. However, it is well known that clinical vitamin D deficiency (see

below), in which the mineralisation of bone is impaired administration of vitamin D restores bone formation (Norman, 2008).

Calcitonin is a peptide of 32 amino acids. It is a hormone secreted by the specialised C cells found in the thyroid follicles. Its actions oppose those of PTH. Calcitonin is able to decrease blood calcium levels by direct inhibition of mediated bone resorption and by enhancing calcium excretion by the kidney (Masi and Brandi, 2007). Calcitonin secretion increases in hypercalcaemia and decreases when plasma Ca^{2+} is low. Circulating concentrations are normally low (<15 pg/mL in males and 10 pg/mL in females) but can be markedly elevated with C cell hyperplasia or medullary thyroid cancer (Verburg et al., 2013). Calcitonin actions are mediated by the calcitonin receptor (CR), a GPCR that couples through multiple G proteins to diverse signal transduction pathways. Different isoforms of calcitonin receptors resulting from alternative splicing of the gene have been described in various animal species with differential tissue expression transcripts and different signalling properties (Moore et al., 1995; Nussenzveig et al., 1994). One of the most important pathways is coupled to the cAMP signal transduction, but calcitonin receptors may also couple to the phospholipase C (PLC) enzyme pathway. Other authors also demonstrated that calcitonin when bound to CTRs stimulates Shc tyrosine phosphorylation of MAPK Erk1/2 (Chen et al., 1998; Pondel, 2000).

Osteoclasts are the major target for the action of calcitonin. Calcitonin is able to interfere with osteoclast differentiation from precursor cells and fusion of mononucleated precursors to form multinucleated cells in bone marrow cultures (Takahashi et al., 1988). Calcitonin may also inhibit several components of the osteoclast function, such as the release of acid phosphatase and the expression of carbonic anhydrase II (Zaidi et al., 1994; Zheng et al., 1994), which is an enzyme responsible for pH regulation, CO_2 and HCO_3^- transport, and maintaining H_2O and electrolyte balance (Sly and Hu, 1995).

Sex steroids also play a key role in the maintenance of bone integrity. In fact, the loss of ovarian function at menopause has long been associated with bone loss (for a review, see Zallone, 2006). In males, although not so abrupt, the

decline of androgen levels has also been implicated to deleterious effects in the skeleton. In fact, it is widely accepted that androgens are locally transformed into oestrogens by aromatase on bone cells (Bilezikian, 2002).

The classical receptors for oestrogens (the ERα and ERβ) or androgens (AR) are expressed in bone marrow stromal cells, osteoblasts, osteoclasts and their precursor cells (Bellido et al., 1995; Couse and Korach, 1999). This suggests that the effects of sex steroids on bone may be mediated directly on such cells (Zallone, 2006). Several studies have indicated that ER receptors are important in the regulation of cytokine production that may act in an autocrine and paracrine manner, regulating osteoclastogenesis and osteoblastogenesis. These may be produced by osteoblasts, stromal cells and lymphocytes (Manolagas and Jika, 1992).

Studies have shown that after menopause, there is an increase on circulating levels of IL-1 and IL-6 (Manolagas et al., 2002). These have been associated with bone loss, and several studies have reported that osteoclastogenesis is associated with increasing levels of these interleukins. Knockout of IL-6 gene in mice prevents bone loss following a decrease of sex steroids (Bellido et al., 1995). Other studies showed that in ovariectomized women and mice there are increases of circulating levels of IL-1 and TNF-α, produced by T cells and blood mononuclear cells (Kitazawa et al., 1994; Pacifici et al., 1991). These have been associated with an increased expression of RANKL by osteoblasts, stimulating the differentiation of osteoclast precursors (Wei et al., 2005). IL-7 levels are also associated with postmenopausal bone loss. In an ovariectomy-induced bone loss model in mice, neutralization of IL-7 completely prevents bone loss and rescues bone formation (Weitzmann et al., 2002). This effect seems to be dependent on T cells, since in T cell-deficiency nude mice, injection of IL-7 failed to induce bone loss (Toraldo et al., 2003).

In addition, in mice models of osteoporosis, ovariectomy causes a dramatic increase in the osteoblast and osteocyte apoptosis. Addition of both oestrogens and androgens prevents such effects, a mechanism that was shown to be mediated by a Src/Shc/ERK signalling cascade (Kousteni et al., 2001). Several

authors have suggested that the protective action of sex steroids on bone is due to a change of the ratio among RANKL, cytokine-inducing osteoclastogenesis and OPG (Zallone, 2006). In previous works, it was shown that in human osteoblastic cells, application of 17β-estradiol is able to promote increases in OPG mRNA in a dose dependent manner (Hofbauer et al., 1999). This protein is able to prevent RANK-RANKL association, preventing osteoclastogenesis, as mentioned above.

Other observation regarding sex steroid decay is the parallel increase in prostaglandin E_2 (PGE_2), which is a potent stimulator of bone resorption in organ culture and osteoclast formation in bone marrow and spleen cell cultures (Li et al., 2002). PGE_2 may enhance bone resorption either by increasing RANKL expression caused by activation of prostaglandin receptors in bone marrow cells (Kanematsu et al., 2000), or due to potentiation of RANK signalling caused by activation of prostaglandin receptors in osteoclast progenitor cells (Ono et al., 2005). It was shown that increasing levels of PGE_2 may be inhibited with the application of oestrogens in *ex vivo* cultures of mouse calvariae from oestrogen deficient animals (Feyen and Raisz, 1987).

Although most of the loss in bone mass caused by oestrogen deficiency is primarily due to enhanced bone resorption, there is also a decrease in bone formation (Chow et al., 1992; Qu et al., 1998). Authors showed that during oestrogen deficiency, there is a decrease of TGF-β (Oursler et al., 1991) and IGF-I (Ernst et al., 1989) expression in osteoblasts, leading to a lower stimulation of osteoblast proliferation and differentiation. Oestrogens are also known to stimulate the expression of type I collagen (Ernst et al., 1989). The opposite effects of oestrogen on osteoblast and osteoclast apoptosis have recently been attributed to different kinetics of ERK phosphorylation, since oestrogen causes a transient phosphorylation of ERK in osteoblasts/osteocytes and a sustained phosphorylation in osteoclasts (Chen et al., 2005). Despite of these findings, the effects of sex steroids on bone are still a matter of extensive debate and more work is needed to clarify the cellular mechanisms involved (for a review, see Lerner, 2006).

There are other key regulating factors on bone remodelling, which will be quoted briefly. Growth hormone is a pituitary polypeptide that does not have direct effects on bone resorption, and its direct actions on bone formation are still controversial. It causes a slight stimulation of IGF-I by skeletal cells and through this local factor, growth hormone may regulate bone formation (see above). In fact, this direct effect on bone cells is of minor importance since these express only a low level of growth hormone receptors. However, growth hormone is known to play an important role in stimulating bone formation *in vivo*, and in particular, during growth, appearing to be necessary for the maintenance of normal bone mass (Canalis, 1993).

Thyroid hormones are known to play a role in normal growth and development, acting primarily on cartilage formation in conjunction with IGF-I. These are able to stimulate bone resorption, with clinical implications. For example, hyperthyroid patients may have hypercalcaemia, and postmenopausal patients on chronic thyroid suppression may be prone to develop osteopenia (Baran and Braverman, 1991).

1.3.3. Bone disorders and therapeutic approaches

On this topic, focus will be given to diseases that affect bone remodelling and some of the respective therapeutic approaches. Special focus will be given to osteoporosis, since it is the most common form of bone disease, afflicting a growing number of people every year. Other less prevalent diseases will be referred briefly, such as renal osteodystrophy, Paget's disease, osteopetrosis, rickets and osteogenesis imperfecta (Table 2).

Osteoporosis, previously mentioned in this work, is a chronic metabolic disease of the bone which is characterized by low bone mass and its structural deterioration. It is the result of factors that negatively affect bone mass and density. Is the most common disease in humans which affects both sexes and all races (Feng and McDonald, 2011; Schuiling et al., 2011). Osteopenia is a condition of lower than normal density and is considered to be a precursor to osteoporosis by some authors, although not everyone with osteopenia develops

osteoporosis (Schuiling et al., 2011). Osteoporosis is traditionally classified into primary and secondary types: the first is further divided into type I and type II osteoporosis (Aaseth et al., 2012). Type I osteoporosis is commonly known as postmenopausal osteoporosis and is a common bone disorder in postmenopausal women which is caused primarily by oestrogen deficiency resulting from menopause (see previous section for details and cell mechanisms of disease). Type II osteoporosis is commonly known as age-related osteoporosis or senile osteoporosis, and it is associated with aging in both women and men (see section 1.2.3 for details).

Table 2. Examples of bone disorders

Osteoporosis
Primary • Menopause associated • Age related Secondary • Glucocorticoid induced • Immobilization induced
Renal osteodystrophy
Paget's disease
Osteopetrosis
Rickets
Osteogenesis imperfecta

Of all of these diseases, osteoporosis is the most common. There are other secondary causes of osteoporosis that were omitted here, focusing on the primary forms of the disease. See text for details.

Secondary osteoporosis is more widely, resulting from secondary complications of various other medical conditions, consequences of deviations in physical activity, diet or therapeutic interventions for certain disorders (Feng and McDonald, 2011). Glucocorticoid-induced osteoporosis was mentioned previously (section 1.2.1) and is a very common cause of bone loss. As already stated, drugs that are commonly used to treat a variety of inflammatory conditions and autoimmune disorders, such as rheumatoid arthritis, asthma, and multiple sclerosis, promote an unbalance between bone formation and resorption. In fact, bone loss begins within the first several months of initiating glucocorticoid treatment, and extended glucocorticoid-based therapy leads to a significant increase in bone fracture (van Staa et al., 2000). Immobilization-induced osteoporosis is also common, and results from a basic principal regarding bone: one of the major functions of bone remodelling is to adapt bone material and its structural properties to the mechanical demands that are placed on the

skeleton, which includes both mechanical loading and weight bearing (Feng and McDonald, 2011). Prolonged bed rest or immobilization resulting, for example, from paralysis or casting a limb reflects a decrease in mechanical requirements, resulting in this form of osteoporosis (Takata and Yasui, 2001). Osteocytes, the mechanical sensors of bone, are often associated to this regulated process of bone adjustments to changes in mechanical loading or weight bearing. Mechanical loading of the skeleton leads to deformation of bones, shear forces and streaming potentials within osteocyte lacunae and canalicular network, which are detected primarily by osteocytes (You et al., 2000). In fact, genetically modified mice lacking osteocytes develop osteoporosis due to defective mechanotransduction (Tatsumi et al., 2007). Osteoblasts and osteoprogenitors are also important mechanosensing cells. They release ATP and other molecules due to shear stress, resulting in increased osteogenesis (Riddle et al., 2007). This topic will be extensively discussed in the following sections. All bone cells should respond somehow to loaded mechanical stress and should have its share in pathogenesis of immobilization-induced bone loss.

Since osteoporosis is a growing problem in developed nations, it is of impaired importance to develop new strategies to overcome this public health issue. Prevention through nutrition and exercise is still the best way to avoid osteoporosis. Regarding the first, calcium and vitamin D are recommended both for the prevention of osteoporotic fractures (for details, see previous section) (Schuiling et al., 2011). Exercise has a deep impact preventing osteoporosis, depending on the type, timing, frequency and duration. It builds bone mass density and changes the size and shape of bone (Hamilton et al., 2010). Although the optimal paybacks of exercise and its effects on peak bone mass density occur prior to menopause, women who are postmenopausal can prevent approximately 2% of bone mass density loss with aerobics, weight-bearing and resistance exercises (Hamilton et al., 2010). Pharmacological treatment, however, is initiated when there is a history of an osteoporotic vertebral or hip fracture, T scores equal or worse than -2.5 at the lumbar spine, femoral neck, or total hip region, or T scores from -1.0 to -2.5, with a risk for a major osteoporotic fracture of at least

20% or a hip fracture of at least 3% within 10 years (given by the FRAX score) (Hamdy et al., 2010). Regarding the pharmacological available treatments for osteoporosis, there are drugs existing that decrease the rate of bone resorption and thereby, slow the rate of bone loss (antiresorptive therapy); other drugs promote bone formation (anabolic therapy). Regarding the antiresorptive agents, the most common are bisphosphonates, calcium supplements, vitamin D and its analogues, selective oestrogen receptor modulators (SERM) and calcitonin (for a review, see Tella and Gallagher, 2014). Bisphosphonates are enzyme-resistant analogues of pyrophosphate, which has a role in regulating bone resorption. Bisphosphonates form tight complexes with calcium in the bone matrix, and are released slowly as bone is resorbed by the osteoclasts, which are thus exposed to high concentrations of the drugs (Rang et al., 2011; Watts and Diab, 2010). Bisphosphonates inhibit bone resorption by an action mainly on the osteoclasts. There are two forms of bisphosphonates: non-nitrogen-containing bisphosphonates and nitrogen containing bisphosphonates. The first group inhibits osteoclastic activity by producing toxic analogues of ATP that cause cell death (Frith et al., 1997). It includes etidronate, clodronate and tiludronate. Their binding affinity and antiresorptive potency differs, since they have different side chains (Watts and Diab, 2010). Nitrogen containing bisphosphonates inhibit an enzyme called farnesyl pyrophosphate synthase, involved in the 3-hydroxi-3-methylglutaryl coenzyme A reductase pathway. Inhibition of this enzyme interferes with prenylation, preventing the addition of 15- and 20-carbon side chains that anchor GTP-binding proteins to osteoclast cell membrane, leading to less resorptive osteoclasts that will enter in apoptosis. These include alendronate, risedronate, ibandronate and zoledronate. Bisphosphonates are usually well tolerated (given weekly and monthly). When given orally, in some cases, they may cause severe oesophageal distress. In some instances, they can be given intravenously for skeletal protection. Possible side effects have been described, mainly related to long-term intake of bisphosphonates, like osteonecrosis of the jaw, musculoskeletal pain, atrial fibrillation, atypical fractures and oesophageal cancer.

For most patients with osteoporosis, however, the benefits of treatment outweigh the risks (for a review, see Watts and Diab, 2010).

Calcium gluconate and calcium lactate are calcium salts used therapeutically, given orally. Studies suggest that supplemental calcium in elderly individuals, usually combined with vitamin D, improves bone mineral density. Dosing varies in the range of 1000 mg/day in adolescents and young adults and 1500 mg/day in the elderly (Varenna et al., 2013). Calcium salts, however, can cause gastrointestinal disturbance among other secondary effects. Calcimimetics are compounds that mimic the action of calcium via CaSR (calcium sensing receptors) to inhibit PTH secretion by parathyroid glands. Subsequent decrease in PTH levels leads to a decreased bone resorption. Cinacalcet is a calcimimetic used for the treatment of secondary hyperthyroidism due to chronic renal disease and for patients with hypercalcaemia associated with parathyroid carcinoma. Hypocalcaemia may be an adverse effect of cinacalcet, so it should not be used in situations where initial plasmatic calcium is less than 8.4 mg/dl (Filopanti et al., 2013).

Calcitonin is a powerful inhibitor of osteoclast activity that acts through specific receptors, preventing the release of acid phosphatase and the expression of carbonic anhydrase II (Zaidi et al., 1994; Zheng et al., 1994) (see previous section). Salmon calcitonin (salcatonin) is the form most often used in human therapy at doses that markedly exceed the endogenous production rate of human calcitonin. It is 50 to 100 times more powerful than human calcitonin (for a review, see Body, 2002). Side effects may include nausea, vomiting, and facial flushing, among others (Rang et al., 2011).

As mentioned in the previous section, during menopause, the decrease in oestrogens has a deep impact on bone formation. One way to ameliorate this condition is to carry out a hormone replacement therapy. Since hormones may act on different tissues, a group of new non-hormonal agents have been developed termed selective oestrogen receptor modulators (SERM). These may exhibit agonist actions on some tissues and antagonist actions on others. An important SERM is raloxifene. This SERM is used clinically in postmenopausal women to

slow bone loss, decreasing bone fracture risk. It supresses osteoclast activity and bone remodelling similarly to oestrogen through high affinity interactions with ERα (Aref et al., 2013; Ettinger et al., 1999). In fact, oestrogens prevented bone loss in ovariectomized rats (Turner et al., 1993). Some authors have also hypothesized that raloxifene may also act directly on the bone matrix to improve material properties, namely affecting tissue-level biochemical properties through non-cell mediated effects on hydration (Gallant et al., 2014). As in other pharmacological approaches, raloxifene may have adverse effects, namely the increased incidence of deep venous thrombosis (Grady et al., 2004).

Regarding the anabolic therapy, PTH and PTH fragments are a new line of compounds that may be used to promote bone formation. The peptide fragment (1-34) is currently in clinical use, the teriparatide. The binding of the ligand to the receptor activates adenylate cyclase and a number of phospholipases (A, C, and D) and increases intracellular levels of cAMP and calcium (Whitfield et al., 1997). Possible cellular mechanisms include the expression of skeletal IGF-I and bone-forming genes. Evidence has shown that teriparatide increases osteoblast growing rate and prevents osteoblastic apoptosis. These processes increase the number of osteoblasts and the rate of new bone formation, and prolong osteoblast survival (for a review, see Misiorowski, 2011). Teriparatide is given subcutaneously once a day. Despite of well tolerated, there has been reported nausea, dizziness, headache and arthralgia. Mild hypercalcaemia, transient orthostatic hypotension and leg cramps have also been reported (Rang et al., 2011).

Some agents have both anabolic and anti-resorptive actions. Such an example is strontium ranelate. This dual mode of action was demonstrated in experimental studies on bone cells and pharmacological studies in animals (for a review, see Neuprez et al., 2008). It seems that strontium decreases differentiation and resorbing activity of osteoclasts and increases osteoclast apoptosis (Marie et al., 2001). In contrast, strontium ranelate seems to enhance pre-osteoblastic cell replication and collagen synthesis in culture (for a review, see Saidak and Marie, 2012). In addition, it modulates the osteoprotegerin/receptor activator of nuclear factor kappa B ligand (OPG/RANKL) system in favour of OPG, and so preventing

osteoclast maturation (Atkins et al., 2009). Finally, strontium ranelate administration decreases bone resorption and maintains bone formation in adult ovariectomized rats, which results in the prevention of bone loss and an increase in bone strength (Morohashi et al., 1995). In addition, clinical studies have shown that strontium ranelate reduces vertebral, nonvertebral and hip fractures over 1-5 years. Its spectrum of activity covers osteopenia and osteoporosis. In elderly subjects, strontium ranelate also promotes a reduction in vertebral and nonvertebral fractures (Neuprez et al., 2008).

Renal osteodystrophy refers to a varied group of metabolic bone diseases that accompany chronic kidney diseases (Feng and McDonald, 2011). Its pathophysiology is complex and reflects PTH and vitamin D importance on bone turnover and related pathological abnormalities. High-turnover renal osteodystrophy can best be categorized as a secondary hyperparathyroidism state and low-turnover disease is characterized by a relative hypoparathyroidism state. Sustained PTH levels increases bone turnover with increased osteoblasts, osteoclasts and osteocytes and a consequent disorder on remodelling leads to abnormal bone formation, increased osteoid and fibrosis. Hypocalcaemia, vitamin D deficiency, hyperphosphataemia are primary factors contributing to secondary hyperparathyroidism (Malluche et al., 2010; Moe et al., 2006). Quantitative histomorphometry in conjunction with appropriate laboratory analyses is often used to make the diagnosis (Moe et al., 2006). Abnormal mineralization reflects the process of calcification of collagen in bone, reflected histologically as abnormal osteoid. This impaired mineralization is still poorly understood, but it’s believed to be caused by vitamin D and/or mineral deficiencies, aluminium toxicity, and possibly to a prolonged acidotic state (Feng and McDonald, 2011).

The most common form of osteodystrophy is characterized by an absolute or relative PTH deficiency accompanied by an extremely low rate of bone formation. Factors implicated in this disorder include increased plasma calcium, vitamin D toxicity, and altered growth factors and cytokines such as BMPs, TGF- β, IGF-I and IL-6 (Hruska et al., 2008; Malluche et al., 2010).

For high-turnover disease, the therapeutic approach is focused on phosphorous restriction or use of phosphate binders, correcting vitamin D deficiency and, occasionally, the use of calcium supplements. Calcimimetic agents are recently being used to inhibit PTH secretion. For low-turnover disease, primary methods are designed to increase PTH levels, usually reducing calcium and vitamin D as well (Feng and McDonald, 2011).

Paget's disease is a bone disorder characterized by a high bone turnover. Paget's disease is an osteoclastic-mediated disorder of bone that results in abnormal bone resorption associated with inadequate remodelling that leads to mechanically weakened bone. It's a disease that is more prevalent with increasing age. Most patients are asymptomatic, but many present bone pain, bone deformities, secondary arthritis, and in some instances, secondary to bone deformities, neurological problems. Although the pathogenesis of the disease is largely unknown, it is believed to be a primary disorder of increased osteoclast bone resorption with a resulting secondary increase in osteoblast activity and new bone formation. Resulting trabecular bone is abnormal with a disorganized appearance (Feng and McDonald, 2011; Singer, 2009). It seems to be a genetic cause, since it follows a familial pattern (Beauregard et al., 2013; Gruener and Camacho, 2014). One such mutation is a gene that encodes an ubiquitin-binding protein that plays a role in NF-kB signalling (Wright et al., 2013). Associated to this disease is chronic viral infection, namely with paramyxoviral and the canine distemper virus (Bianco et al., 1992; Gordon et al., 1991). The therapeutic approach is directed at inhibiting osteoclasts, usually using bisphosphonates; in alternative, calcitonin is used to both prevent osteoclast activity and as analgesic, since it helps to treat patients with substantial bone pain; additionally, therapy may include surgery in order to correct bone deformities (Siris, 1995).

Osteopetrosis are that family of congenital diseases which is characterized by a strikingly radiopaque skeleton, with loss of distinction between cortex and marrow space. The hallmark of the disease is the persistence of "cartilaginous bars" deep within metaphyseal and diaphyseal bone. Such islands of devitalized cartilage surrounded by bone represent the residue of nonresorbed primary spongiosa, a

component of endochondral ossification (Teitelbaum, 1993). The forms of disease are as follows: severe and intermediate autosomal recessive types and a milder autosomal dominant subtype. There have been identified several gene mutations all which encode proteins that involve osteoclast-mediated bone resorption. The three most important mutations are related to carbonic anhydrase II, proton pump and chloride channel. The proton pump is abundantly expressed in the ruffled membrane and transports protons into the resorption lacuna to create and maintain a low pH (~4.5); this situation provides a high concentration of acid onto a strongly basic mineral to dissolve the inorganic component of bone hydroxyapatite; these protons are generated from carbon dioxide and water by carbonic anhydrase II; in the other hand, the chloride channel, also present in the ruffled membrane, transports Cl^- into the resorption lacuna to maintain electron neutrality. Mutations in these indicated proteins will compromise bone resorption, leading to different forms of osteopetrosis (Feng and McDonald, 2011). This disease is diagnosed through the use of a combination of clinical and radiological parameters; increased serum concentrations of creatine kinase BB isozyme and tartrate-resistance acid phosphatase (which derive from osteoclasts) are often observed (Bollerslev et al., 2000; Waguespack et al., 2002). In terms of therapy, which is only supportive, haematopoietic stem cell transplantation is usually a choice (Driessen et al., 2003); treatment regimens with interferon-γ, calcium restriction, vitamin D, steroids and PTH have all been tried, with no apparent success (Feng and McDonald, 2011).

Rickets is a pathological situation in which there is a reduction of mineralisation of newly-formed bone, that is, osteoid is unmineralised (osteomalacia) and endochondral calcification at the growth plate is absent or reduced, with associated growth-plate deformity (Elder and Bishop, 2014). Vitamin D deficiency is the most common cause in most cases. It remains unclear whether an absolute threshold for vitamin D exists, below which rickets is inevitable; rickets can also occur when vitamin D is within the range associated with maximum calcium absorption, but calcium intake is low (DeLucia et al., 2003). In some cases, abnormalities that mainly affect phosphate metabolism or bone-tissue

mineralisation might be the cause. The consequences of vitamin D deficiency in bone are the direct inhibition of osteoblast progenitors and an increase in RANKL expression, with a parallel decrease in OPG, decreasing bone formation and enhancing bone turnover. Treatment is focused on fixing the vitamin D deficiency, increasing sunshine exposure and through dietary supplements (such as ergocalciferol). Some uncommon forms of rickets have been reported that could not be corrected with vitamin D therapy, which seems to be an inheritable form of the disease, eventually related to vitamin D receptor abnormalities and to vitamin D metabolism anomalies (Elder and Bishop, 2014).

Osteogenesis imperfecta is an inherited connective tissue disorder of remarkable clinical variability, caused by a quantitative or qualitative defect in collagen synthesis and is characterised by bone fragility (Antoniazzi et al., 2000). It is often fatal *in utero* but those who survive fall into a spectrum of skeletal dysfunction ranging from little or no deformity to relentless crippling associated with hundreds of fractures. Cardinal manifestations are low-bone mass and reduced bone mineral strength, leading to bone fragility and deformity. Cortical bone is more diminished than is trabecular bone, and remodelling is typically brisk in this disease (Teitelbaum, 1993). In about 90% of individuals with the clinical diagnosis of osteogenesis imperfecta, mutations in the *COL1A1* and *COL1A2* genes are responsible for the disorder, which code for the α1(I) and α2(I) side chains of type I collagen (Rohrbach and Giunta, 2012). Three types of treatment are available: nonsurgical management (physical therapy, rehabilitation, bracing and splinting), surgery, and drugs to increase the strength of bone and decrease the number of fractures. These include bisphosphonates administered to children with osteogenesis imperfecta, with the rationale that bones with increased volume of osteogenesis imperfecta-quality matrix will be more fracture resistant (Rauch et al., 2006). Other approaches are being considered, as antibodies directed against RANKL, in order to shift the RANKL/OPG ratio, and decrease osteoblast signalling that normally stimulates osteoclast development (Forlino et al., 2011; Hussar and Stevenson, 2010).

In general, the development of new therapeutic approaches is of major importance since these bone disorders, like osteoporosis, are highly prevalent and in increasing incidence on an ageing population. In the next chapter, purinergic signalling, in the context of bone remodelling, will be explored, reinforcing the huge and growing potential of such area in the development of new therapeutic strategies to overcome bone deterioration in common bone-related disorders.

1.4. Purinergic signalling and bone remodelling

1.4.1. Purinergic receptors

As mentioned previously, bone turnover is a complex and finely tuned process. The importance of systemic and local factors has already been stated, and to these we must include the significant role of nucleotides, such as adenosine 5'-triphosphate (ATP) and its derivatives (like adenosine). It is known that nucleotides play fundamental roles in energy metabolism, nucleic acid synthesis and enzyme regulation (Burnstock and Knight, 2004). Nucleotides, predominantly ATP, are present in intracellular concentrations between 2-5 mM, and extracellular concentrations are low, mainly due to the presence of ubiquitous ecto-nucleotidases and other ecto-phosphatases that rapidly hydrolyse extracellular nucleotides to their respective nucleoside 5'-di- and monophosphates, nucleosides and free phosphates or pyrophosphates (see below). Nucleotides may be released from cells via three major mechanisms: 1) cytosolic ATP release from sites of tissue and cell damage (including sites of bone injury), 2) exocytic release, and 3) via intrinsic plasma membrane channels or pores in the absence of cytolysis, which includes controlled release through hemichannels (for a review, see Novak, 2003; for further details, see below). Once released, nucleotides and their derivatives may act on purinergic receptors, which are important (yet largely unknown) bone turnover regulators (Orriss et al., 2010).

The receptors for purines and pyrimidines are classified into two groups: P1 receptors (A_1, A_{2A}, A_{2B}, A_3), which are primarily activated by adenosine, and P2 receptors, which respond to ATP, adenosine diphosphate (ADP), uridine

triphosphate (UTP), uridine diphosphate (UDP) and nucleotide polyphosphates. The P2 receptors may be further subdivided into P2X ligand-gated ion channels and P2Y G-protein-coupled receptors (Abbracchio and Burnstock, 1994; Burnstock and Kennedy, 1985). To date, seven P2X receptors (P2X1-7) and eight P2Y receptors ($P2Y_{1,2,4,6,11-14}$) have been identified, cloned and characterized, displaying different tissue distribution and pharmacological properties (Burnstock, 2007; Orriss et al., 2010). The P2X channels are assembled (in a homo- or heteromeric manner) from seven subunits. Based on agonist efficacy, elctrophysiological properties and desensitization characteristics, P2X receptors have been grouped into three distinct classes (Dubyak, 2007): 1) includes P2X1 and P2X3 receptors exhibiting high affinity for ATP (EC_{50} = 1 μM), which are rapidly activated and desensitized; 2) includes P2X2, P2X4, P2X5, and P2X6 receptors, which have lower affinity for ATP (EC_{50} = 10 μM) and show slow desensitization rate and sustained depolarizing currents; 3) is represented by the homomeric P2X7 receptor, which has very low affinity for ATP (EC_{50} = 300–400 μM). This receptor shows little or no desensitization and acts as a nonselective ion pore (Di Virgilio, 1995), like other P2X receptors (Williams and Jarvis, 2000). Besides acting as an ATP-gated ion channel, prolonged activation of the P2X7 receptor favour permeation of plasma membrane to high-molecular weight (up to 900 Da) hydrophilic molecules, such as ATP and glutamate. P2Y and P1 receptors are classical seven-transmembrane domain receptors couped to G-proteins and numerous intracellular second messengers, including cAMP and inositol (1,4,5)-triphosphate (IP3) cascades (Burnstock and Verkhratsky, 2010).

1.4.2. P2 receptor expression by osteoblasts

Previous studies confirmed that osteoblasts and osteoblast-like cells (from human and rodent species) express P2 receptors and that extracellular nucleotides could transiently increase $[Ca^{2+}]_i$ and induce IP3 formation (Orriss et al., 2010). Table 3 illustrates the growing body of work around P2 receptors in these cells. Data so far obtained strenghten the role of these receptors in osteoblast function. After the pioneering work of Kumagai and coworkers showing

that ATP plays a central role in bone physiology (Kumagai et al., 1991), numerous studies demonstrated that nucleotides influence osteoblast and osteoclast function through the activation of both P2X and P2Y receptors. There are, however, conflicting results in the literature regarding the predominant role of P2 receptors on bone remodelling that derive mostly from heterogeneity of P2 receptors expression among osteoprogenitors vs differentiated cell populations, cell lines vs primary cell cultures (see *e.g*., Wesselius et al., 2011), and the existence of striking differences on the molecular composition and activity of these receptors between human and rodent species (see *e.g*. Roger et al., 2010). Another confounding factor that authors often disregard, which must be considered of clinical relevance, is the influence of systemic factors, such as the hormonal condition at bone microenvironment at the time of isolation of the cells. Another constrain is on what concerns the predominant use of animal models and immortalized cell lines, and less on non-modified human cells (Table 3). To obscure the pharmacological characterization and the investigation of the relative importance of P2 purinoceptors in bone biology, most studies had to deal with a lack of commercially-available selective agonists and/or antagonists. Nevertheless, data show that P2 receptors have some role on osteoblast cells function.

For instance, P2X1 and P2X3 receptors were shown to be involved in the prevention of bone nodule mineralization of osteoblast cultures from neonatal Sprague–Dawley rats, since P2X1 and P2X3 receptor agonists, α,β-meATP and β,γ-meATP, inhibited bone mineralisation without affecting collagen production (Orriss et al., 2012).

The activity of the P2X2 receptor seems to be more relevant in osteoclasts, where it increased bone resorption. Resorption increased 5.6-fold when osteoclasts from neonatal rats were cultured for 26 h on ivory discs in the presence of ATP, with a maximum effect occurring at relatively low concentrations (0.2-2 μM) (Morrison et al., 1998). The osteoclast promoting action of ATP was greatly amplified when these cells were cultured in acidified media (pH 6.9-7.0) (King et al., 1997).

Table 3. P2 receptor expression by osteoblasts

Receptor	Species	Cell type	Transduction mechanisms	Proposed function and signalling	References
P2X1	Rat	Primary	Cation channel (Ca^{2+} and Na^{+})	Involved in bone mineralization inhibition (rat osteoblasts)	Orriss et al., 2012; Orriss et al., 2013
P2X2	Rat	Primary	Cation channel (Ca^{2+})	Unknown. Also expressed in osteoclasts, involved in bone resorption (rodent osteoclasts)	Alqallaf et al., 2009; Hoebertz et al., 2000; Morrison et al.,1998; Nakamura et al., 2000
	Human	MC3T3-E1, SaOS-2			
P2X3	Rat	Primary	Cation channel	Involved in bone mineralization inhibition (rat osteoblasts)	Orriss et al., 2012
P2X4	Rat	Primary	Cation channel (Ca^{2+})	Involved in pore formation (human osteoblast-like cells)	Alqallaf et al., 2009; Ihara et al., 2005
	Human	SAM-1, MG-63, SaOS-2			
P2X5	Rat	Primary	Cation channel	Involved in human osteoblast like cell's differentiation – stimulation of the MAP kinase pathway	Hoebertz et al., 2000; Ihara et al., 2005; Nakamura et al., 2000; Orriss et al., 2006
	Human	MC3T3-E1, SAM-1			
P2X6	Rat	Primary	Cation channel	Largely unknown; regulator of MSC commitment	Ihara et al., 2005; Zippel et al., 2012
	Human	SAM-1			
P2X7	Rat	Primary	Cation channel, large pore after prolonged activation	Controversial: Apoptosis (human cells); induction of membrane cell blebbing and increased bone formation - LPA and PGE_2 synthesis (newborn rat osteoblasts); skeletal mechanotransduction (mouse osteoblasts); also expressed in human osteoclasts, involved in cellular apoptosis and cytoskeleton rearrangements	Alqallaf et al., 2009; Gartland et al., 2001; Jorgensen et al., 2002; Ke et al., 2003; Li et al., 2005; Nakamura et al., 2000; Orriss et al., 2006; Orriss et al., 2010 ; Panupinthu et al., 2007; Panupinthu et al., 2008
	Human	MG-63, SaOS-2, Primary			
	Mouse	Primary			

Table 3. *(Continued)* P2 receptor expression by osteoblasts

$P2Y_1$	Rat	Primary	G_q/G_{11} PLCβ activation	Modulate osteoblast responses to PTH – increased *c-fos* expression (human and rat osteoblasts)	Bowler et al., 1999; Buckley et al., 2001; Hoebertz et al., 2000; Maier et al., 1997; Orriss et al., 2006
	Human	MG-63			
$P2Y_2$	Rat	Primary	G_q/G_{11}, possibly G_i/G_0, PLCβ activation	Propagation of intracellular Ca^{2+} waves (human osteoblasts); inhibition of bone mineralization – inhibition of ALP (rat osteoblasts); stimulation of Erg1 and Runx-2 expression – activation of PKC and ERK pathways (osteoblast-like HOBIT cell line); sensitises mechanical stress-activated Ca^{2+} channels – activation of ERK, p38 MAPK and JNK1 pathways (ROS-A 17/2.8 osteoblastic cells)	Bowler et al., 1995; Costessi et al., 2005; Hoebertz et al., 2000; Hoebertz et al., 2002; Jorgensen et al., 2000; Katz et al., 2006; Katz et al., 2008; Maier et al., 1997; Orriss et al., 2006; Orriss et al., 2007; Pines et al., 2003
	Human	MG-63, Primary, SaOS-2, Te85			
$P2Y_4$	Rat	Primary	G_q/G_{11}, possibly G_i, PLCβ activation	Possibly involved in the inhibition of cell mineralization (rat osteoblasts)	Hoebertz et al., 2002; Maier et al., 1997; Orriss et al., 2006
	Human	MG-63			
$P2Y_6$	Rat	Primary	G_q/G_{11} PLCβ activation	Largely unknown; also expressed in osteoclasts, involved in their survival (rat osteoclasts)	Korcok et al., 2005; Maier et al., 1997; Orriss et al., 2006
	Human	MG-63			
$P2Y_{11}$	Human	osteosarcoma HOS cells	G_q/G_{11}, G_s	Largely unknown	Liu and Chen, 2010
$P2Y_{12}$	Rat	Primary	G_i, inhibition of adenylate cyclase	Involved in bone nodule mineralization	Orriss et al., 2010; Syberg et al., 2012
$P2Y_{13}$	Rat	Primary	G_i/G_0	Increased ALP activity and osteogenic differentiation (mouse bone marrow stromal cells)	Biver et al., 2013; Orriss et al., 2011
$P2Y_{14}$	Rat	Primary	G_q/G_{11}	Unknown, although suggest to play a role in early osteogenic differentiation of human MSCs	Maier et al., 1997; Orriss et al., 2006; Zippel et al., 2012

Growing number of studies indicate that extracellular nucleotides play a role in modulating osteoblast function, via P2 receptors. Alkaline phosphatase, ALP; c-jun NH2-terminal protein kinase 1, JNK1; extracellular related kinase, ERK; lysophosphatidic acid, LPA; Mesenchymal stem cells, MSCs; p38 mitogen-activated kinase, p38 MAPK; phospholipase A_2, PLA_2; phospholipase D, PLD; prostaglandin E_2, PGE_2; protein kinase C, PKC (based on indicated references; see text for details).

The P2X4 receptor is also expressed in osteoblasts, where it is involved in membrane pore formation. In human osteoblast-like cells, the prototypic P2X7 receptor agonist, BzATP, caused the incorporation of YO-PRO 1 fluorescent dye (a strong indicator of pore formation), but selective P2X7 receptor antagonists were unable to completely block pore formation, thus suggesting that coexpressed P2X4 may also contribute to this mechanism (Alqallaf et al., 2009).

ATP, acting on P2X5 receptors (among others), has been reported to stimulate osteoblast proliferation (Nakamura et al., 2000). In human osteoblast-like MG-63 cells, Nakamura and co-workers showed that extracellular ATP increased [^{3}H]thymidine incorporation and cell proliferation, a mechanism that seemed to be MAP kinase-dependent (Nakamura et al., 2000).

The P2X6 receptor's role is osteoblast-like cells is largely unknown, yet it has been shown to be expressed in primary cultures of humans and rats (Ihara et al., 2005).

Regarding the P2X7 receptor, a huge number of studies suggested that this receptor plays important roles in distinct cellular mechanisms, but not all of them agree on the pathways involved. Gain of function polymorphisms of the P2X7 receptor have been associated with increased bone mass (Jorgensen et al., 2012), whilst loss of function polymorphisms have been associated with increased fracture risk, reduced bone mineral density and osteoporosis in postmenopausal women (Gartland et al. 2012; Husted et al., 2013; Ohlendorff et al., 2007). It was also suggested that the increased vertebral fracture incidence could be due to an increased number of osteoclasts. However, heterogeneity of the P2X7 receptor expression among osteoprogenitors and differentiated osteoblast-like cell populations has also been a matter of concern (Burnstock et al., 2013). The P2X7 receptor has been implicated in reversible morphological changes (*e.g*. blebbing) (Li et al., 2005; Panupinthu et al., 2007) and/or in the promotion of apoptosis in osteoclasts (Burnstock, 2002; North, 2002). However, controversy still exists about its significance in humans that are undermined by striking species differences shown for the P2X7 receptor (see *e.g*. Roger et al., 2010).

Regarding the P2Y metabotropic receptors, some authors have suggested some of their particular roles in osteoblast cells. The $P2Y_1$ receptor was shown to modulate human osteoblast responses to systemic factors such as PTH. These authors have shown that nucleotide agonists such as ADP and ATP increased $[Ca^{2+}]_i$ and moderately induced the expression of the *c-fos* proto-oncogene. They also observed that there is a synergistic effect on *c-fos* induction with the combination of ATP and PTH, previously referred as a key bone cell regulator. They concluded that extracellular ATP, via P2Y receptors, can potentiate strong responses to ubiquitous growth and differentiating factors (Bowler et al., 1999).

Regarding the $P2Y_2$ and $P2Y_4$ receptors, Hoebertz and co-workers showed that ATP and UTP at ≥ 1 μM are strong inhibitors of mineralization in cultured primary rat calvarial osteoblasts (Hoebertz et al., 2002). The potent inhibitory actions of ATP and UTP were consistent pharmacologically with mediation via the $P2Y_2$ and $P2Y_4$ receptor subtypes, although reactive blue 2, a $P2Y_4$ receptor antagonist, failed to prevent the nucleotide-induced blockade of mineralization. This suggests that the $P2Y_2$ receptor is mainly involved in this mechanism, an idea that was also supported by other authors (Orriss et al., 2007). In fact, it was shown that $P2Y_2$ knockout mice presents huge increases in trabecular and cortical bone in both femora and tibiae, through skeletal analysis by dual energy X-ray absorbtiometry and micro-CT (Orriss et al., 2007). Other interesting reports have shown that the $P2Y_2$ receptor activation is coupled to intracellular pathways, such as PKC, with subsequent stimulation of Runx-2 protein expression (Costessi et al., 2005). The p38 mitogen-activated protein kinase and c-Jun NH2-terminal protein kinase (JNK) were also shown to function downstream the $P2Y_2$ receptor activation; such intracellular pathways were associated with mechanical-stress activated Ca^{2+} channels (Katz et al., 2006; Katz et al., 2008).

UDP-sensitive $P2Y_6$ receptors have been poorly explored. In fact, uracil nucleotides seem to be released in parallel to ATP by different cell types as soluble factors in response to mechanical stimulation (see for a review, *e.g.*, Hoebertz et al., 2003). Previous works have shown that UDP-sugars, like UDP-glucose ($P2Y_{14}$ receptor activator) may be released together with ATP under

resting and stimulating conditions (Kreda et al., 2007, 2008; Lazarowski et al., 2003). UDP itself is an end product during glycogen synthesis, which may be released to the extracellular fluid. In fact, using an enzymatic assay, others detected UTP release from several cell types with a proportion of approximately 10-30% of released nucleotides (Lazarowski and Harden, 1999). UTP can also act through P2Y receptors to upregulate ATP release from human osteoblasts, providing a possible feedback mechanism (Bowler et al., 1998). Human osteosarcoma cell lines also express mRNAs for $P2Y_4$ and $P2Y_6$ receptors (Maier et al., 1997), which are preferentially or selectively activated by UTP and UDP, respectively. Curiously, rat osteoclasts also express $P2Y_6$ receptors which seem to be involved in their survival, a cell mechanism dependent on NF-kB activation (Korcok et al., 2005). Decreased bone resorption was observed in $P2Y_6$ knockout mice, thus suggesting that this receptor may play an important role in bone remodelling (Orriss et al., 2011).

The $P2Y_{11}$ receptor expression was detected by RT-PCR in a human osteosarcoma cell line, the HOS cells, but its function remains elusive. It is usually coupled to $G_{q/11}$ and G_s, increasing IP3 levels and adenylate cyclase function, respectively (Liu and Chen, 2010).

$P2Y_{12}$ receptors are usually associated to bone nodule formation. This association came with the use of clopidogrel (Plavix), a selective $P2Y_{12}$ receptor antagonist that is widely prescribed to reduce the risk of heart attack and stroke and acts via the inhibition of platelet aggregation. Using rat osteoblasts, researchers found that clopidogrel was able to inhibit *in vitro* mineralization in a concentration-dependent manner, slowing osteoblast proliferation and cell viability, together with a reduction in ALP activity and collagen formation. *In vivo*, clopidogrel-treated mice showed a decreased trabecular bone volume in the tibia and femur (Syberg et al., 2012) when compared to control mice, reinforcing this receptor's role in bone formation. More studies are required to investigate the role of the $P2Y_{12}$ receptor in human bone remodelling before one can assume that its antagonists, like clopidogrel, are deleterious to the maintenance of bone mass.

Regarding $P2Y_{13}$ receptors, recent studies demonstrated a decreased bone formation and bone resorption in a knockout mice, suggesting a role of these receptors in bone remodelling (Orriss et al., 2011). In fact, recent studies demonstrated that in mice, ADP stimulation of $P2Y_{13}^{+/+}$ (but not $P2Y_{13}^{-/-}$) adherent bone marrow stromal cells (BMSCs) increased the formation of alkaline phosphatase-colony-forming units (CFU-ALP), as well as the expression of osteoblastic markers, namely osterix, alkaline phosphatase, and type I collagen (Biver et al., 2013). In the knockout model, BMSCs were shown to preferentially differentiate into adipocytes, with a higher expression of PPAR*γ*2 and adipsin (Biver et al., 2013).

Concerning the $P2Y_{14}$ receptor, although its role in bone remodelling remains elusive, some authors suggested that, together with P2X6 and $P2Y_4$, it seems an important regulator in MSC commitment to both adipogenic and osteogenic differentiation of human adipose tissue-derived stem cells. They found that $P2Y_{14}$ receptor's expression decreased from undifferentiated to osteoblast-differentiated cells and suggested that its role in bone cells should be important at an early stage of differentiation (Zippel et al., 2012).

1.4.3. Release of nucleotides from osteoblast and osteoblast-progenitor cells

Extracellular adenine nucleotides, like ATP, play important roles in the differentiation and function of osteoblasts. Both BMSCs and differentiated osteoblasts constitutively release ATP; the amount of released ATP increases dramatically when these cells are submitted to mechanical stress or in pathological conditions, such as during hypoxia and inflammation (Brandao-Burch et al., 2012; Orriss et al., 2009). Their specific targets include several subtypes of P2Y (G-protein coupled) and P2X (ligand-gated ion channels) purinoceptors (Romanello et al., 2005). Romanello first described controlled ATP release with mechanical stimulation (Romanello et al., 2001). Several studies point towards exocytosis being the primary mechanism by which ATP is released from osteoblasts (Genetos et al., 2005; Orriss et al., 2009; Romanello et al., 2005). However,

several other studies suggest that maxi-anion channels (Sabirov and Okada, 2009), connexins, namely connexin 43 (Thi et al., 2012), and the P2X7 receptor (Brandao-Burch et al., 2012), are also important in the release of ATP from osteoblasts. Pannexins have also been implicated in nucleotide release, namely pannexin 1 and 3 (Ishikawa et al., 2014; Thi et al., 2012). In this respect, pannexin 1, in concert with P2X7 receptors, have been suggested to form a complex that underlies the hemichannel function in osteoblasts mechanosignalling involving the release of ATP. Connexin 43-null osteoblasts have unaltered mechanically-induced PGE_2 release and ATP-induced YoPro dye uptake. In parallel, the same authors found that PGE_2 release in response to fluid shear stress was abolished in P2X7 receptor-null osteoblasts; likewise, ATP-induced fluorescent dye uptake was attenuated following treatment of wild-type cells with a P2X7 receptor antagonist or a pannexin 1 channel blocker (Thi et al., 2012). Regarding pannexin 3, this hemichannel is the most abundant in bone (as well as in skin and cartilage) (Shestopalov and Panchin, 2008). Authors have suggested that it plays a key role in the transition from cell proliferation to cell differentiation. Using rat primary calvarial cells and explants it was found that the pannexin 3 hemichannel inhibits cell growth by promoting β-catenin degradation through GSK3β activation and inhibit cyclin D1 transcription and Rb phosphorylation through reduced cAMP/PKA/CREB signalling. Furthermore, the pannexin 3 may be located in the endoplasmic reticulum, where it induces the transcription and phosphorylation of p21 through the calmodulin/Smad pathway, resulting in the cell cycle exit (Ishikawa et al., 2014).

The mechanisms responsible by the release of ATP and, therefore, the concentration of the nucleotide in the extracellular milieu depend on the differentiation status of the cell, with mature bone-forming cells releasing up to sevenfold more ATP than immature proliferating cells (Orriss et al., 2009). In this regard, Riddle and co-workers have shown that BMSCs release ATP through exocytosis. They also showed that ATP was a prerequisite for fluid flow–induced increases in intracellular calcium concentration, activation of calcineurin, nuclear translocation of NFATc1, and proliferation (Riddle et al., 2007). Other authors have

shown that the expression of connexin 43, the most predominant connexin expressed by bone cells, increases with osteoblastic differentiation, possibly allowing gap junction hemichannels to augment in prevalence and increasing their functional importance in bone cell mechanotransduction (Donahue, 2000).

Many studies have shown increased extracellular levels of ATP in response to external stimuli, namely hypoxia (Orriss et al., 2009), mechanical stress (Hecht et al., 2013) and fluid flow (Genetos et al., 2005; Romanello et al., 2001; Rumney et al., 2012). In the case of hypoxia, this stimulus resulted in an increased ATP release from osteoblasts up to 2.5-fold without affecting cell viability, a mechanism that was found to be mainly driven by exocytosis (Orriss et al., 2009). Hecht and co-workers mechanically induced ATP release from osteoblast cells (MC3T3-E1) using a stretching device integrated into scanning electrochemical microscopy. They showed that stretching MC3T3-E1 cells up to 21% results in a concentration of ~30 μM of extracellular ATP, which was almost abolished in the presence of nifedipine, an L-type voltage sensitive calcium channel (L-VSCC) inhibitor (Hecht et al., 2013). Fluid flow was shown to involve extracellular-signal regulated kinase (ERK1/2) activation, a mechanism that is Ca^{2+}-, PKC and ATP-dependent in MC3T3-E1 osteoblasts (Liu et al., 2008b). Additionally, this ATP-dependent ERK1/2 phosphorylation was shown to be mediated through P2X7 receptors (Liu et al., 2008b).

Ultrasound stimulation (US) was also found to promote ATP release from osteoblasts, providing a basis to interpret the beneficial effect of US to accelerate fracture healing. Authors found increased concentrations of ATP in culture medium of US-treated cells and both ATP and US stimulation caused increased receptor activator of nuclear factor-kappa B ligand (RANKL), decreased osteoprotegerin expression and increased cell proliferation by SaOS-2 cells (Hayton et al., 2005). Furthermore, low-intensity pulsed ultrasound stimulation (LIPUS) treatment was shown to induce ATP release from osteoblasts. This resulted in an increased $P2Y_1$-dependent proliferation (Alvarenga et al., 2010).

Calcitropic hormones were found recently to promote ATP release from osteoblast-like cells. Vitamin D3, in the absence of mechanical stimulation,

increased ATP release from SAOS-2 and ROS 17/2.8 osteoblasts when compared to the control stiuation (Biswas and Zanello, 2009). These authors found that ATP secretion was abolished when cells are preincubated with inhibitors of vesicular exocytosis. Similarly, siRNA VDR (vitamin D receptors) silencing prevented vitamin D3 stimulation of ATP exocytosis (Biswas and Zanello, 2009). Furthermore, bisphosphonates, which are used for the treatment of osteoporosis (see section 1.3.3), have been reported to induce ATP release, favouring P2Y receptors activation. Authors showed that risedronate promotes non-lytic ATP release leading to activation of ERKs through the involvement of P2Y receptors, namely $P2Y_1$ and $P2Y_2$. In fact, bisphosphonates were previously shown to exert part of their effects by using a sort of membrane-receptor triggering by means of connexin 43 direct binding, due to their charged nature (Plotkin et al., 2002).

These and other studies suggest that the local ATP release in response to different stimuli may represent a local trigger in bone that may influence osteogenesis (Burnstock et al., 2013).

1.4.4. ATP hydrolysis: ecto-nucleotidases and osteoblasts

Once released, nucleotides may be rapidly broken down enzymatically by a cascade of a number of ecto-nucleotidases (Burnstock et al., 2013) (Figure 4). Consequently, ectonucleotidases may command a series of physiological responses by regulating P2 receptor activation (Kukulski et al., 2005). There are four families of ecto-nucleotidases: the NTPDases (ecto-nucleoside triphosphate diphosphohydrolase), the NPPs (ecto-nucleotide pyrophosphatase/ phosphodiesterase), alkaline phosphatases and ecto-5'-nucleotidase (Zimmermann et al., 2012). Ecto-nucleotidases may have overlapping specificities. For instance, NTPDases catalyse the reactions NTP → NDP + phosphate (Pi) and NDP → NMP + (Pi), whereas NPPs hydrolyse NTP → NMP +

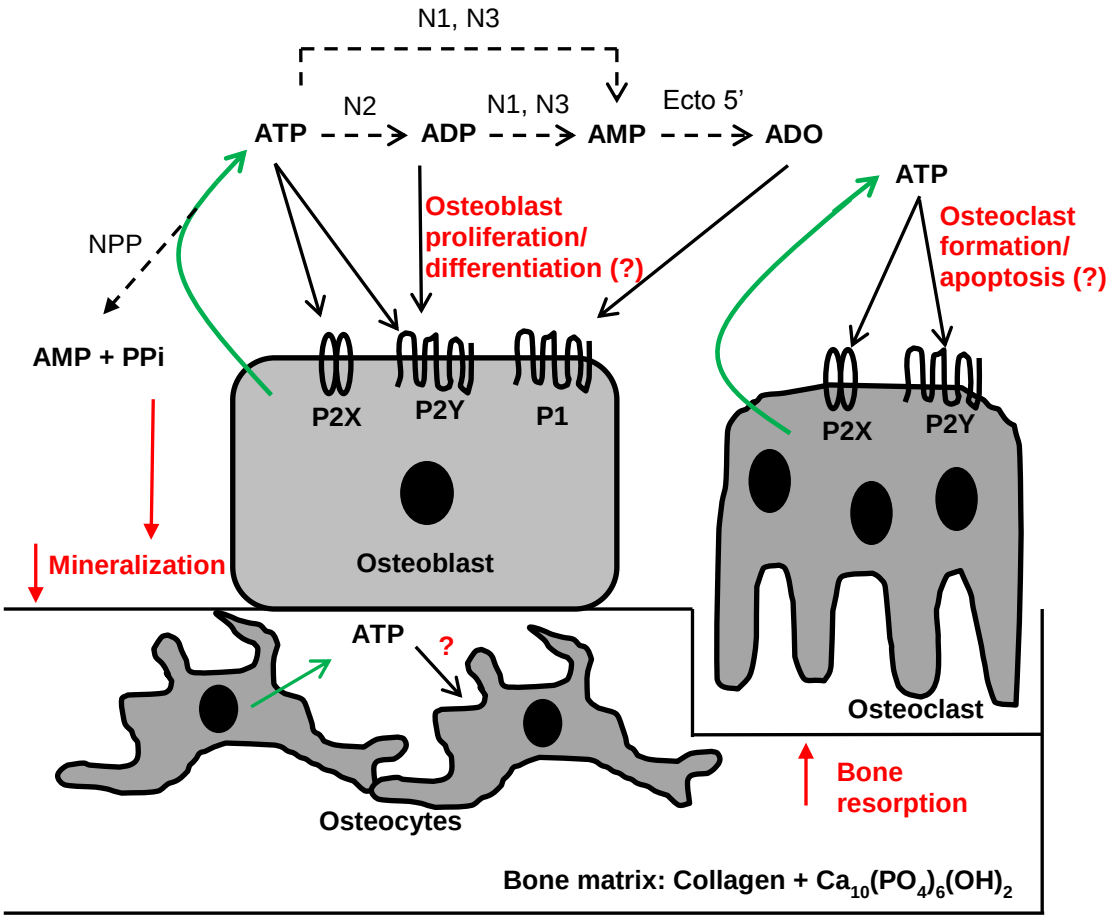

Figure 4. Schematic overview of functional effects of ATP on bone cells. ATP release from osteoblasts, osteocytes and osteoclasts can influence their function in an autocrine or paracrine manner, influencing both bone resorption and formation. Black arrows represent P1 or P2 activation, dashed arrows represent ATP breakdown and red texts/arrows represent multiple ATP effects on bone cells. NTPDase1, N1; NTPDase2, N2; NTPDase3, N3; ecto 5'-nucleotidase, Ecto 5'; nucleotide pyrophosphatase/phosphodiesterase, NPP; largely unknown or controversial effect, ?. Figure adapted from Burnstock et al., 2013.

pyrophosphate (PPi) or NDP → NMP + Pi (Burnstock et al., 2013). Regarding the NTPDase family, these are dominantly ectonucleotidases (Zimmermann et al., 2012). Four of the eight members of this family, namely NTPDase1, NTPDase2, NTPDase3 and NTPDase8 appear to be relevant to the control of P2 receptor signalling since they are located at the outer surface of the plasma membrane and hydrolyse nucleotides in the range of concentration that activates P2 receptors (Bigonnesse et al., 2004; Lavoie et al., 2004; Mateo et al., 1999; Picher et al., 1996; Smith and Kirley, 1999). These enzymes have two plasma membrane

spanning domains with an active site facing the extracellular milieu (Zimmermann et al., 2012). In contrast, the other NTPDases, namely 4-7, are anchored to the membrane of intracellular organelles by one (NTPDases5 and -6) or two (NTPDases4 and -7) transmembrane domains and their catalytic site faces the lumen of intracellular compartments, namely the Golgi apparatus and the endoplasmatic reticulum (Kukulski et al., 2005; Trombetta and Helenius, 1999; Wang et al., 1998). Since NTPDases have different biochemical properties, they are able to regulate P2 receptor activation differently, since they have different abilities in dephosphorylating nucleoside triphosphates and diphosphates (exclusively in the presence of divalent cations, Ca^{2+} or Mg^{2+}, and usually all active within a pH of 7.0-8.5) (Kukulski et al., 2005). Among the four plasma membrane bound NTPDases, NTPDase1 (also known as CD39) hydrolyses ATP and ADP with the same affinity, NTPDase2 is a preferential triphosphonucleosidase whereas NTPDase3 and 8 are functional intermediates between NTPDase1 and -2 (Kukulski et al., 2005). NTPDase1, -2, -3 and -8 efficiently hydrolyse ATP and UTP with *Km* values in the micromolar range, indicating that they should terminate the effects exerted by nucleotide agonists at P2X1-7 and $P2Y_{2,4,11}$. In fact, Kukulski and co-workers, using COS-7 transfected cells, showed that NTPDase1 does not allow the accumulation of ADP, suggesting that it should terminate the activation of $P2Y_{1,12,13}$ receptors. In contrast, they showed that NTPDases2, -3 and -8 are expected to promote the activation of ADP receptors, because in the presence of ATP, they produce a sustained (NTPDase2) or transient (NTPDases3 and -8) accumulation of ADP (Kukulski et al., 2005). Additionally, they found that all plasma membrane NTPDases dephosphorylate UTP with a significant accumulation of UDP, favouring $P2Y_6$ receptor activation. Furthermore, comparing *Km* and *Vmax* values obtained from combined ATP and UTP hydrolyses experiments, authors revealed that adenine nucleotides are better substrates than uracil nucleotides for human NTPDase1 and -2. Furthermore, they found that human NTPDase3 dephosphorylated ADP to AMP much faster than UDP to UMP, leading to a sustained UDP accumulation (Kukulski et al., 2005). So, differential

expression and function of these NTPDases will dictate which P2 receptors will be preferentially activated within a cell population (Figure 4).

Previous studies have revealed the coexistence of both ATP consuming and ATP-generating activities (nucleoside diphosphokinase or ecto-NDPK) on the cell surface, namely in human SaOS-2 osteoblast-like cells (Buckley et al., 2003). In addition, the expression of multiple NTPDases and NPPs has been previously reported (Orriss et al., 2007; Orriss et al., 2010). Regarding NPPs, NPP1 has been found to be important in bone biology. PPi, mostly generated from ATP hydrolysis by NPP1, is a strong inhibitor of bone mineralization (for a review, see Mackenzie et al., 2012). In fact, several studies suggest that ATP is a primary source of PPi in bone, pointing to a dual inhibitory action on bone mineralization via both P2 receptors mediated signalling and direct hydrolysis to PPi (Figure 4). It is believed that PPi is important to prevent ectopic mineralization (for example in soft tissues) (see *e.g*. Nitschke et al., 2012). At the same time, PPi is a source of Pi which is important in hydroxyapatite formation, a conversion that is undertaken by ALP. So, the concerted action of these two enzymes, NPP1 and ALP, is an important aspect of bone mineralization process.

In this regard, it was found that, in a mouse model lacking NPP1, the trabecular number, trabecular bone volume, structure model index, trabecular and cortical thickness are all significantly reduced in tibiae and femurs from NPP1 ($^{-/-}$) mice when compared to control animals, showing that NPP1 ($^{-/-}$) mice are characterized by severe disruption to the architecture and mineralization of long-bones, dysregulation of calcium/phosphate homeostasis, all suggesting an important role for this enzyme in bone remodelling (Mackenzie et al., 2012). Other studies suggested that generalised arterial calcification in infants and severe hypophosphataemia is associated with recessive inactivating mutations in the ENPP1 gene (see *e.g.* Rutsch et al., 2003). One should be aware that NPPs may be inhibited by AMP, which is one of the end products of ATP hydrolysis. Intriguingly, it binds with more affinity when compared to ATP (Landt and Butler, 1978; Stefan et al., 2005). This aspect raises the question if NPP's action has a sustained impact on bone remodelling *in vivo*, particular in a circumstance in which

ecto-5'-nucleotidase activity is compromised. In such case, AMP accumulation would lead to NPP1 inhibition, reducing ATP catalysis by this enzyme.

ATP breakdown throughout the enzymatic cascade leads, eventually, to adenosine production by ecto-5'-nucleotidase. Adenosine has been shown to be produced by human osteoblast cells via ATP breakdown, modulating the secretion of IL-6 and osteoblastogenesis (Costa et al., 2011; Evans et al., 2006). In fact, human bone marrow derived MSCs were shown to express ecto-5'-nucleotidase (CD73) and to express all four P1 receptors (Costa et al., 2011). Agonists for all four receptors concentration-dependently increase MSCs proliferation. In particular, authors showed that A_{2B} activation with 5'- (N-ethylcarboxamide)adenosine (NECA) facilitates osteogenic differentiation measured by increases in ALP activity; the effect of NECA was prevented with the selective A_{2B} antagonists, PSB 603 (Costa et al., 2011). These results were strenghten by the use of A_{2B} knockout mice, thus revealing the importance of this receptor subtype in osteogenic differentiation of mesenchymal stem cells and bone formation *in vivo* (Carroll et al., 2012). Regarding ecto-5'-nucleotidase (CD73) as an important enzyme on adenosine formation by osteoblasts, a recent study demonstrated that CD73 knockout mice show osteopenia, with significant decreases of osteoblast-cell markers. In the same study, *in vitro* experiments revealed that CD73 deficiency results in impaired osteoblast differentiation, but not in the number of osteoblast progenitors. In addition, authors demonstrated the enhanced expression of osteocalcin and bone sialoprotein in MC3T3-E1 cells overexpressing CD73 (Takedachi et al., 2012). These and other studies reinforce the role of P1 receptors on bone remodelling.

1.4.5. P2 receptor expression by osteocytes and osteoclasts

So far, focus has been given to purinergic signalling in osteoblast progenitor cells, osteoblasts and, only briefly, to osteoclasts. Besides their role in bone formation, P2 receptors have been previously pointed as important modulators of bone resorption. Briefly, it was found that ATP was able to stimulate resorption by cells from human osteoclastoma (Bowler et al., 1998) and from rodent osteoclasts

(Morrison et al., 1998). It was later suggested that these pro-resorptive effects were mediated by the P2X2 receptors. ADP, via $P2Y_1$ receptors, was also pointed as an important receptor involved in osteoclast formation and activity, since ADP and 2-methylthioADP at nanomolar to submicromolar levels caused up to four to six-fold increases in osteoclastic bone resorption (Hoebertz et al., 2001). Interestingly, $P2Y_1$ receptor knockout animals showed reduced trabecular bone in long bones, which pointed this receptor as an important modulator of bone remodelling (Orriss et al., 2011). However, another receptor was subsequently shown to mediate ADP effects on osteoclasts. $P2Y_{12}$ receptor is expressed in osteoclasts (Orriss et al., 2011), and KO of this receptor impaires ADP elicited responses by these cells (Su et al., 2012).

The $P2Y_6$ receptor seems also to be important in osteoclast survival. Its activation on cultured osteoclasts prevents TNF-α-induced apoptosis, also promoting NF-kB translocation and activation (Korcok et al., 2005).

Regarding P2X7 receptor, its activity appears to be complex in osteoclasts. It was demonstrated that ATP release from osteoclasts is P2X7 mediated (Brandao-Burch et al., 2012), and that blockade of the pore-forming P2X7 receptor inhibits formation of multinucleated human osteoclasts *in vitro* (Gartland et al., 2003). It was further demonstrated that P2X7 activation may lead to NF-kB (Korcok et al., 2004) and PKC translocation (Armstrong et al., 2009), cytoskeletal reorganization and secretion of lytic granules into the resorption lacunae (Hazama et al., 2009). However, others have shown that ATP release in response to mechanical stimuli may act on P2X7 receptors to inhibit osteoclast resorption (Naemsch et al., 2001).

Regarding P1 receptors, it was suggested that adenosine resulting from released ATP may act on P1 receptors from osteoprogenitor cells, inducing IL-6 secretion and inhibition of osteoprotegerin release, promoting osteoclastogenesis (Evans et al., 2006). The A_1 receptor was pointed as a promoter of osteoclast differentiation, since blockade of this receptor resulted in disruption of the association of tumor necrosis factor receptor-associated factor 6 (TRAF6) and transforming growth factor-β-activated kinase 1 (TAK1), a signalling event that is important for NF-κB activation (He and Cronstein, 2012). A_{2A} receptors seem to be

equally involved in osteoclast function. Studies point for a compromised osteoclast differentiation upon A_{2A} activation (Mediero et al., 2012). Others have shown that P2X7-mediated ATP release and subsequent adenosine formation may increase osteoclast fusion, an effect that seems to be A_{2A} mediated (Pellegatti et al., 2011).

Within the osteoblast lineage, osteocytes are also important cells within the bone, since they represent 90% of all bone cells (see section 1.3.1). However, little is known about the role of purinergic signalling in their survival or function (Figure 4). Part of the difficulty to study these cells is that 3D-cultures are needed to allow the maintenance of their phenotype. As previously mentioned, they form an interconnected cell network located in the fluid-filled lacunocanaliculi system, allowing them to translate mechanical signals into biochemical signals to effector cells, facilitating bone remodelling (Burnstock et al., 2013). Using cell cultures, it was possible to realize that mechanical stimulated osteocytes could present $[Ca^{2+}]_i$ transients that are reduced by both suramin and thapsigargin, suggesting that this is due to ATP acting via P2Y receptors (Huo et al., 2008). Osteocytes were also found to express T-type voltage-sensitive calcium channels, and its $\alpha_2\delta_1$ subunit was shown to regulate mechanical-induced release of ATP (Thompson et al., 2011). Some authors suggest that ATP release from osteocytes (Kringelbach et al., 2014) may act *in vivo* to reduce progressive, age-related mineral encroachment from the surrounding bone, thus preventing cell death and cell "fossilization" (Burnstock et al., 2013) (Figure 4). In fact, in MLO-Y4 osteocytes, P2X7 and $P2Y_2$ receptors were found to be functionally expressed (Kringelbach et al., 2008).

Current evidence shows that purinergic signalling exerts complex local effects on the function of bone cells (*e.g*. osteoblasts). The effects of this signalling system are influenced by multiple factors including the receptor subtype, the extracellular nucleotides present locally and the expression of ecto-nucleotidases which will, in turn, regulate nucleotides accumulation and activity. However, there is still a huge controversy around the importance of each of the purinergic

receptors and enzymes on bone cells and how they may interact to promote bone remodelling

Since purinergic signalling has now been implicated in many bone disorders (for a review, report to Burnstock et al., 2013), it is of great importance to explore these potential targets for future therapies, clarifying the molecular mechanisms operating upstream and downstream of these receptors in both health and disease. These unexplored cell targets will certainly prove useful in the therapeutic management of bone diseases in the future.

2. GOALS

The specific aims of the project were to investigate:

(1) The expression and function of uracil nucleotide-sensitive receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) in human MSCs, since their role in osteogenic differentiation was largely unknown;

(2) The expression and function of P2X7 receptors on osteogenic differentiation of human MSCs in culture, exploring the underlying molecular mechanisms involved; this was done due to the controversy of the P2X7 receptor role on bone remodelling in human cells;

(3) The relevance of NTPDases in the management of osteogenic differentiation and/or cell proliferation in younger females *versus* postmenopausal women; this topic is, so far, largely unknown, particularly in non-modified human cells.

Hopefully, this will provide new insights for development of novel therapeutic strategies for bone disorders such as osteoporosis, in which targeting both receptors and ecto-nucleotidases may prove beneficial.

3. ORIGINAL RESEARCH PAPERS

The results obtained in this thesis were published / submitted for publication as original research papers, as follows:

- Paper 1: **Noronha-Matos JB**, Costa MA, Magalhães-Cardoso T, Ferreirinha F, Freitas R, Neves JM, Sévigny J and Correia-de-Sá P. (2012). Role of ecto-NTPDases on UDP-sensitive $P2Y_6$ receptor activation during osteogenic differentiation of primary bone marrow-derived mesenchymal stem cells from postmenopausal woman. *Journal of Cellular Physiology,* 227, 2694-2709.
  DOI: 10.1002/jcp.23014. PMID: 21898410;

- Paper 2: **Noronha-Matos JB**, Coimbra J, Sá-e-Sousa A, Rocha R, Marinhas J, Freitas R, Gomes-Guerra S, Ferreirinha F, Costa MA and Correia-de-Sá P. (2014). P2X7-induced zeiosis promotes osteogenic differentiation and mineralization of postmenopausal bone marrow-derived mesenchymal stem cells. *FASEB Journal, Epub ahead of print.*
  DOI: 10.1096/fj.14-257923, PMID: 25169056

- Paper 3: **Noronha-Matos JB**, Calejo I, Magalhães-Cardoso MT, Silva I, Ferreirinha F, Rocha R, Marinhas J, Freitas R, Costa MA, Pelletier J, Sévigny J and Correia-de-Sá P. (2014). Inhibition of NTPDase3 on bone marrow-derived mesenchymal stem cells may be a novel therapeutic strategy to increase bone formation in postmenopausal women**.** *In Preparation.*

Paper 1

Journal of Cellular Physiology. 2012. 227, 2694-2709.

DOI: 10.1002/jcp.23014. PMID: 21898410.

Role of ecto-NTPDases on UDP-sensitive $P2Y_6$ receptor activation during osteogenic differentiation of primary bone marrow-derived mesenchymal stem cells from postmenopausal women

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ABSTRACT

This study aimed at investigating the expression and function of uracil nucleotide-sensitive receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) on osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs) in culture. Bone marrow specimens were obtained from postmenopausal female patients (68±5 years old, *n*=18) undergoing total hip arthroplasty. UTP and UDP (100 μM) facilitated osteogenic differentiation of the cells measured as increases in alkaline phosphatase (ALP) activity, without affecting cell proliferation. Uracil nucleotides concentration-dependently increased $[Ca^{2+}]_i$ in MSCs; their effects became less evident with time (7>21 days) of the cells in culture. Selective activation of $P2Y_6$ receptors with the stable UDP analogue, PSB 0474, mimicked the effects of both UTP and UDP, whereas UTPγS was devoid of effect. Selective blockade of $P2Y_6$ receptors with MRS 2578 prevented $[Ca^{2+}]_i$ rises and osteogenic differentiation caused by UDP at all culture time points. MSCs are immunoreactive against $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. While the expression of $P2Y_6$ receptors remained fairly constant (7~21 days), $P2Y_2$ and $P2Y_4$ became evident only in less proliferative and more differentiated cultures (7<21 days). The rate of extracellular UTP and UDP inactivation was higher in less proliferative and more differentiated cell populations. Immunoreactivity against NTPDase1, -2, and -3 rises as cells differentiate (7<21 days). Data show that uracil nucleotides are important regulators of osteogenic cells differentiation predominantly through the activation of UDP-sensitive $P2Y_6$ receptors coupled to increases in $[Ca^{2+}]_i$. Endogenous actions of uracil nucleotides may be balanced through specific NTPDases determining whether osteoblast progenitors are driven into proliferation or differentiation.

INTRODUCTION

Dynamic mechanical loading increases bone density and strength by promoting osteoblast proliferation, differentiation, and matrix production. The molecular mechanisms through which mechanical forces are converted into biochemical signalling in bone are still poorly understood. There is increasing evidence pointing to extracellular nucleotides, such as ATP and UTP, as soluble factors released in response to mechanical stimulation in different cell systems (see for a review, *e.g.*, Hoebertz et al., 2003). Nucleotides are also released from cells as a consequence of pathological insults, like hypoxia and inflammation. Mechanisms of nucleotides release including vesicle exocytosis, ATP-binding cassette transporters, connexin hemichannels, and voltage-dependent anion channels, have been considered (Burnstock, 2006). Once released, the action of nucleotides may be rapidly terminated by cell-surface-located ectonucleotidases (Yegutkin, 2008; Zimmermann, 2000). Therefore, nucleotides released to the bone microenvironment form concentration gradients enabling differential targeting of receptors to produce selective special effects. For instance, rapid breakdown of ATP into adenosine restricts its action to that of a localized signal and shifts purinergic transmission mediated by nucleotide-sensitive P2 receptors to long-lasting modulatory signals mediated by metabotropic P1 adenosine receptors. Adenine nucleotides working through several subtypes of P2 purinoceptors regulate all aspects of bone biology including development, growth, turnover, and repair (for a revision, see Orriss et al., 2010). Recently, we provided evidence that adenosine is also an important regulator of osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs) through the activation of subtype-specific (A_1, A_{2A}, and A_{2B}) receptors depending on the proliferation status of the cells (Costa et al., 2011).

Besides the well-recognized role of ATP in bone remodelling, few reports have questioned the action of uracil nucleotides as autocrine/paracrine signalling molecules in the human bone. So far, constitutive release of UTP has been reported for several cell types, but not yet for osteoblasts. Nevertheless, UTP can easily be generated extracellularly from other nucleotides through the action of

ecto-enzymes (Lazarowski et al., 2000; Zimmermann, 2000). UTP can also act through P2Y receptors to upregulate ATP release from human osteoblasts, providing a possible feedback mechanism (Bowler et al., 1998). Localization of metabotropic $P2Y_2$ receptors that recognize both ATP and UTP as the most potent agonists was evidenced by *in situ* hybridization in human osteoblasts (Bowler et al., 1995). Human osteosarcoma cell lines also express mRNAs for $P2Y_4$ and $P2Y_6$ receptors (Maier et al., 1997), which are preferentially or selectively activated by UTP and UDP, respectively. Interestingly, this receptor profile varies between primary cells and established osteoblastic cell lines (reviewed by Bowler et al., 2001). To add further complexity, there is a clear heterogeneity of P2 receptors expression within single populations of both primary and clonal osteoblasts (see *e.g.*, Dixon et al., 1997). In rat BMSCs, intracellular Ca^{2+} mobilization via UTP-sensitive $P2Y_2$ receptors was stronger at high cell density, indicating that cell density closely regulates cell cycle progression through increased $P2Y_2$ receptors expression as cells proliferate (Ichikawa and Gemba, 2009). Likewise, Orriss et al. (2006) showed that ATP and UTP induced Ca^{2+} transients in primary rat osteoblasts increase during differentiation with time in culture; these changes were notably accompanied by an increase in the expression of P2Y receptors, particularly the UTP-sensitive $P2Y_2$ receptor and to a lesser extent the $P2Y_4$ and $P2Y_6$ receptors. Follow-up studies demonstrated that both ATP- and UTP-treated rat osteoblasts deposited abundant collagenous matrix with the characteristic morphology of bone nodules (Orriss et al., 2010). Elevated Ca^{2+} levels can activate a variety of intracellular signalling systems in different cell types. The question is which ones are activated in human osteoprogenitor cells upon P2Y receptors stimulation by uracil nucleotides and, most importantly, whether receptor expression and coupling to specific signalling pathways also change due to postmenopausal and/or pathological bone modifications?

Although the above observations are intriguing, the relative abundance of each receptor subtype and their exact role regarding bone formation and remodelling during the osteogenic differentiation in human osteoprogenitor cells remains unclear. Dissection of individual receptor responses has been hampered by the

lack of specific P2Y receptor agonists and antagonists, along with the presence of ecto-nucleotidases bound to the extracellular side of osteoblast membranes that rapidly interconvert or degrade nucleotides. Four plasma membrane-bound members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family have been cloned, namely NTPDase1, -2, -3, and -8 (Robson et al., 2006; Zimmermann, 2000). These enzymes have distinct biochemical properties. NTPDase1 hydrolyzes ATP/UTP and ADP/UDP equally well, NTPDase2 preferentially hydrolyzes triphosphonucleosides, and NTPDase3 and -8 have intermediate hydrolysis profiles (Kukulski et al., 2005; Stefan et al., 2005; Zimmermann, 2000). The hydrolysis of tri- and diphosphonucleosides by NTPDases yields AMP/UMP as final products that can be fully dephosphorylated to adenosine/uridine by ecto-5'-nucleotidase (Colgan et al., 2006). This enzyme (also known as CD73 membrane-cell marker), together with CD105 and CD90 antigens, has been widely used to identify osteoblast progenitors derived from human BMSCs (Dominici et al., 2006).

Comprehensive studies on the kinetics of the metabolism of uracil nucleotides via ecto-nucleotidases in human osteoprogenitor cells are still lacking. In spite of this, the coexistence of various metabolic pathways represents an opportunity for regulating cell-specific responses mediated by the P2-receptor family to locally produced nucleotides (see for a review, Kukulski et al., 2011). In this work, we characterized the extracellular enzymatic pathways responsible for the catabolism of uracil nucleotides along osteogenic differentiation of human primary bone marrow-derived MSCs in culture using HPLC analysis. In addition, we investigated the time course of expression of NTPDase subtypes (1, 2, and 3) and uracil-sensitive P2 purinoceptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) by confocal microscopy. First passage human MSCs obtained from postmenopausal female patients (68±5 years old) undergoing total hip arthroplasty were used. Cell cultures were characterized for proliferation (MTT assay) and osteogenic differentiation (measured as increases in alkaline phosphatase activity); to this end, cells where cultured for 28 days in the absence and in the presence of UTP, UDP, and several subtype-selective receptor agonists and antagonists. The ability of uracil

nucleotides to trigger intracellular $[Ca^{2+}]_i$ transients via $P2Y_2$, $P2Y_4$, and $P2Y_6$ with respect to the growth state of primary MSCs (7 vs. 21 days in culture) was evaluated by confocal microscopy using the fluorescent Ca^{2+} indicator, Fluo-4NW. Hopefully, our findings will contribute to elucidate the role of extracellular uracil nucleotides (UTP and UDP) as important targets for local regulation of osteoblast cell proliferation and differentiation in the postmenopausal bone, prompting for new strategies to control disorders where bone destruction exceeds bone formation (*e.g*., osteoporosis, rheumatoid arthritis, fracture mal-union).

MATERIALS AND METHODS

Cell Cultures

Human bone marrow specimens were obtained from the neck of the femur of selected adult female patients (68±5 years old, *n*=18) undergoing total hip arthroplasty as a result of primary osteoarthrosis. For comparison purposes we also used bone marrow specimens from younger adult female patients of 14–40 years old (*n*=4) (Figure 6). Informed consent to use the biological material, that would be otherwise discarded, was obtained. The procedures were all approved by the Ethics Committees of Centro Hospitalar de Vila Nova de Gaia—Espinho (University Hospital) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of the University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki. Bone marrow samples were placed immediately in fresh-frozen α-minimal essential medium (α-MEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (standard culture medium) and transported to the laboratory on the day or following day of surgery. Bone marrow cells were dispersed on plastic dishes by repeated gently pipetting, cultured in standard culture medium, and incubated at 37^0C in a humidified atmosphere of 95% air and 5% CO_2. Non-adherent cells were removed after 5 days and after that the culture medium of the adherent cells was changed twice a week. Primary cultures were maintained for 10–15 days until near confluence when adherent

cells were enzymatically released with 0.04% trypsin–EDTA solution and 0.025% type I collagenase in phosphate-buffered saline (PBS), at pH 7.4 during 15–20 min. The resultant cell suspension was cultured (10^4 cells/cm^2) (day 0) in conditions known to favour osteogenic differentiation. To this end, the standard culture medium was supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone. MSC cultures were established for 28 days in the absence (control) or in the presence of purinoceptor agonists/ antagonists that were added to the culture medium at day 1 (see *e.g.*, Costa et al., 2011). Drugs were renewed in the culture at each medium change, i.e., twice a week. All the experiments were performed in the first subculture, since previous results showed that serial passage of bone marrow-derived MSCs result in the progressive loss of the osteoblast phenotype (Coelho et al., 2000; Fernandes et al., 1997). Cell cultures were routinely monitored by phase contrast microscopy and characterized at days 1, 4, 7, 14, 21, and 28 for cell viability/proliferation (MTT assay), alkaline phosphatase (ALP) activity, and total protein content.

Cell viability/proliferation

MTT assay and total protein content. Proliferation studies included MTT assay and total protein content. MTT assay consisted of the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan reaction product by viable cells. In the last 4 h of each test period, cells were incubated with 0.5 mg/ml of MTT in the conditions referred above. The medium was carefully removed, decanted, and the stained product dissolved with DMSO before absorbance (A) determination at 600 nm using a microplate reader spectrometer. Results were expressed as A/cm^2 (Figure 6A). Total protein content was determined by Lowry's method, after treatment of the cell layer with 0.1 M NaOH for 1 h. Bovine serum albumin (BSA) was used as a standard, and absorbance evaluated at 750 nm. Results are expressed as µg/cm^2 (Amaral et al., 2002; Costa et al., 2011).

Fluorimetric DNA determination. The total cell DNA content was determined as previously described (West et al., 1985). At the end of the culture period, cells

were washed twice with PBS 1X and incubated for 20 min with EDTA (10 mM, pH=12.3) at 37^0C. Cell plates were then kept on ice and pH adjusted to 7.0 with addition of KH_2PO_4 (1 M). To each sample, a solution of Hoescht 33258 (200 ng/ml in NaCl 100 mM plus Tris 10 mM buffer, pH=7.0) was added. Fluorescence was measured (Perkin Elmer, Luminescence Spectrometer LS 30, Waltham, MA) and DNA concentration determined using a DNA calibration curve (0–100 µg DNA/ml). DNA standards were prepared using salmon DNA at 1 mg/ml in Tris (10 mM) plus EDTA (1 mM) buffer (pH=8.0). Results were expressed as µg of DNA/cm^2.

Alkaline phosphatase (ALP) activity

ALP activity was determined in cell lysates (obtained by treatment of the cell layers with 0.1% Triton X in water) and assayed for the hydrolysis (30 min at 37^0C) of *p*-nitrophenyl phosphate (25 nM) in an alkaline buffer solution (pH=10.3), followed by colorimetric determination of *p*-nitrophenol at 405 nm. Results were expressed in nanomoles of *p*-nitrophenol produced per µg of protein (nmol min^{-1} µg $protein^{-1}$) (Amaral et al., 2002; Costa et al., 2011) (Figure 6B). ALP activity is a good indicator for osteoblast cell differentiation as previously shown (Costa et al., 2011; Hoemann et al., 2009).

Histochemical staining for ALP

Fixed cultures (1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, 10 min) were stained for ALP. Fixed cells were incubated for 1 h in the dark with a mixture prepared in Tris buffer (pH=10) containing 2 mg/ml of fast blue RR salt; the incubation was stopped by rinsing the samples with water. The presence of ALP was identified by a brown to black staining, according to the enzyme content (see *e.g.*, Figure 6D). Stained culture wells were photographed using an inverted microscope (Olympus IX8, Tokyo, Japan) coupled to a high-sensitivity digital colour camera (ColorView II, Olympus, Tokyo, Japan) and analysed with the software Cell F (Olympus, Tokyo, Japan).

Kinetic experiments and HPLC analysis

The kinetics of inactivation of UTP and UDP in human primary MSC cultures was studied at days 7 and 21, at 37^0C (three replicas were performed in each individual experiment). The kinetics of extracellular ATP catabolism was also studied for comparison purposes (see *e.g*., Magalhães-Cardoso et al., 2003). After a 30-min equilibration period, cells were incubated with 100 µM of ATP, UTP, or UDP added to the culture medium in the conditions referred above (zero time). Samples (75 µl) were collected from each well at different times up to 30 min for high-performance liquid chromatography (HPLC, LaChrom Elite, Merck, Frankfurt, Germany) analysis of the variation of substrate disappearance and product formation. Aliquots of 20 µl of collected samples were used for nucleotide analysis. The rate of ATP disappearance and subsequent formation of ADP, AMP, and adenosine was analysed as previously described (Costa et al., 2011). Separation of UTP, UDP, UMP, and uridine was achieved by ion-pair reverse-phase HPLC, at room temperature, on a Merck Lichrospher® 100 RP-18 (5 µm) column. The composition of the mobile phase was 60 mM KH_2PO_4 and 5 mM tetrabutylammonium (pH=6) in methanol. Each run consisted of a linear gradient from 5 to 35% methanol (v/v) performed at 1.5 ml/min flow rate during 8 min; re-equilibration of the column required an additional 7-min period. Nucleotides and nucleosides were detected by UV absorption at 262 nm. Under these experimental conditions, the retention times for uracil nucleotides and nucleosides were as follows: UTP (6.41 min), UDP (4.67 min), UMP (2.18 min), and uridine (1.49 min). The actual concentrations of UTP, UDP, UMP, and uridine were expressed in micromolar.

Concentrations of the substrate and products were plotted as a function of time (progress curves). The following parameters were analysed for each progress curve: half-degradation time of the initial substrate, time of appearance of the different concentrations of the products, concentration of the substrate or any product remaining at the end of the experiment. Because enzymatic activity is usually represented as a function of the total protein content, yet in osteoblast differentiating cultures type I collagen accounts to 85–90% of the organic matrix, here we also decided to normalize the ecto-nucleotidase activity by the amount of

viable cells given by the MTT assay. The spontaneous degradation of uracil nucleotides at 37^0C in the absence of the cells was negligible over 30 min. At the end of the experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity. The negligible activity of LDH in the samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental period.

Single-cell $[Ca^{2+}]_i$ transients by confocal microscopy

Human primary MSCs were seeded into 35 mm dishes at a density of $2x10^4$ cells/ml and allowed to grow for 7 or 21 days in supplemented α-MEM medium. On the day of the experiment, cells were washed twice with PBS and incubated at 37^0C for 45 min with the cell-permeant fluorescent Ca^{2+} indicator, Fluo-4NW (2.5 µM), in PBS containing 2.5% pluronic acid in 100 µM DMSO. After removal of the fluorophore loading solution, cells were washed twice more and 150 µl of PBS was added per culture dish. Culture dishes were then mounted on a thermostatized perfusion chamber at the stage of an inverted laser-scanning confocal microscope (Olympus FV1000, Tokyo, Japan) equipped with a 20x magnification objective lens (LUCPLFL 20x PH; N.A. 0.45). From this time onwards, the chamber was perfused continuously (1 ml/min) with gassed (95% O_2 plus 5% CO_2) Tyrode's solution (pH=7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, and glucose 11.2, at 37^0C. Test drugs were delivered using a multichannel perfusion system (ValveLink 8.2, Digitimer, San Francisco, CA). Changes in fluorescence of the Fluo-4NW dye were detected in the time-lapse mode with the FluoView Advanced Software (Olympus, Tokyo, Japan). Fluo-4NW was excited with the 488 nm line a Multi-line Ar laser. The emitted fluorescence was detected at 510–560 nm using the scanner of the confocal microscope (Olympus FV1000, Tokyo, Japan). The fluorescence images were collected at 20 sec intervals. Intracellular Ca^{2+} transients induced by uracil nucleotides were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response) (Henriksen et al., 2006; Panupinthu et al., 2007).

Antibody production

The development and specificity of anti-human nucleotidase antibodies has been reported previously (*e.g.*, Dranoff et al., 2004; Munkonda et al., 2009). Hartley guinea pigs and New Zealand rabbits were obtained from Charles River Laboratories (Quebec City, Canada). Genetic immunization protocol was carried out with plasmids (pcDNA3 for human NTPDase1 and pcDNA3.1 for human ecto-5'-nucleotidase) encoding each protein using New Zealand rabbits for antibodies against human NTPDase1 and Hartley guinea pigs for human ecto-5'-nucleotidase antibodies.

Immunofluorescence confocal microscopy

MSCs were allowed to grow in chamber slides for 7 or 21 days. At the end of each test period, cultured cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 min, washed three times in PBS (10 min each) and, subsequently, incubated with blocking buffer I (10% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN_3) for 1.5 h. Primary antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN_3), were applied [NTPDase1 1:150 (hN1-9_LI4, rabbit), NTPDase2 1:200 (hN2-Kw3I_4, rabbit), NTPDase3 1:200 (hN3-B3_S, mouse), ecto-5'-nucleotidase 1:300 (h5'NT-$2_C I_4$, guinea-pig), $P2Y_1$ 1:50 (goat), $P2Y_2$ 1:150 (rabbit), $P2Y_4$ 1:75 (rabbit), $P2Y_6$ 1:75 (rabbit), P2X7 1:75 (rabbit), osteocalcin 1:75 (rabbit), collagen type I 1:50 (rabbit)] and the slides incubated in the dark for 2 h. After incubation, cells were washed three times in PBS 1x (10 min each); Alexa Fluor 488 (anti-rabbit), Alexa Fluor 568 (anti-mouse), and Alexa Fluor 653 (anti-goat) secondary antibodies in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X) were applied for 1 h. A last wash was performed with PBS 1x and glass slides mounted with VectaShield medium and stored at 4^0C. Observations were performed and analysed with a laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan) (Alqallaf et al., 2009).

Flow cytometry analysis

Plastic-adherent human bone marrow-derived MSCs (first subculture) were allowed to grow for 7 days in conditions known to favour osteogenic differentiation. At the end of this period, the cells were enzymatically released as mentioned before. The resultant cell suspension was centrifuged at 2000 rpm (5 min); the supernatant was discarded and cells were re-suspended in 2ml of PBS 1x containing 0.2% BSA. This procedure was repeated twice. Samples (100 µl) containing 250–500x10^3 cells were incubated for 15 min at room temperature with 10 µl of antigen-specific anti-human (mouse) fluorochrome-conjugated monoclonal antibodies (mAb). The antibodies employed were the following: Anti-CD14 labelled with fluorescein isothiocyanate (FITC) (clone MφP9), anti-CD29 labelled with fluorescein isothiocyanate (FITC) (clone TS2/I6), anti-CD34 labelled with allophycocyanin (APC) (clone 8GI2), anti-CD45 labelled with peridinin-chlorophyll protein (PerCP) (clone 2DI), anti-CD105 labelled with phycoerythrin (PE) (clone IG2), anti-CD117 labelled with phycoerythrin (PE) (clone 95C3); in some experiments, anti-ecto-5'-nucleotidase (h5'NT-2cI4, guinea-pig) was used, combined with the secondary antibody Alexa Fluor 649 (anti-guinea-pig). Cells were first identified based on their flow cytometric characteristics—high sideward (SSC) and forward (FSC) light scatter profile. Cells were then gated on the basis of sideward scatter distribution. Once identified, cells were analysed for the expression of the surface antigens recognized by the mAb referred above. Controls were performed by incubating the cells with the conjugates or secondary antibodies alone, in order to measure unspecific fluorescence (negative controls). All experiments were done in triplicate on a FACSCalibur cytometer (BD Biosciences, San Jose, CA), using the CellQuest software version 3.1 (BD Biosciences, San Jose, CA) for sample acquisition and the Paint-a-gate Pro software (BD Biosciences, San Jose, CA) for data analysis.

Reagents and antibodies

Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine (ADO), adenosine 5'-diphosphate sodium salt (ADP), adenosine 5'-triphosphate disodium salt (ATP), 4-[[4-formyl- 5-hydroxyl-6-methyl-3-

[(phosphonooxy)methyl]-2-pyridinyl]azo]- 1,3-benzenedisulfonic acid tetrasodium salt (PPADS), uridine, uridine 5'-diphosphate trisodium salt (UDP), and uridine 5'-triphosphate trisodium salt (UTP) were purchased from Sigma-Aldrich (St. Louis, MO). 3-[[5-(2,3-Dichlorophenyl)- 1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079), 2'-deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS 2179), N,N''-1,4-butanediyl-bis-[N'-(3-isothiocyanatophenyl) thiourea (MRS 2578), 3-(2-oxo-2-phenylethyl)-uridine-5'-diphosphate disodium salt (PSB 0474), and uridine-5'-(γ-thio)-triphosphate trisodium salt (UTPγS) were obtained from Tocris Cookson Inc. (Bristol, UK). All primary antibodies used in this study have previously been validated: Anti-$P2Y_1$ was from Santa Cruz (Santa Cruz, CA); anti-$P2Y_2$ and anti-$P2Y_4$ were from AbCam (Cambridge, UK); anti-$P2Y_6$ and anti-P2X7 were purchased from Alomone (Jerusalem, Israel); anti-osteocalcin and anti-Type I collagen were from AbD Serotec (Kidlington, Oxford, UK); anti-CD29-FITC (clone TS2/I6) was supplied by eBioscience (San Diego, CA); anti-CD117-PE (clone 95C3) and anti-CD105-PE (clone IG2) were supplied by Immunotech (Marseille, France); anti-CD14-FITC (clone MφP9), anti-CD45-PerCP (clone 2DI), and anti-CD34-APC (clone 8GI2) were supplied by BD Biosciences, San Jose, CA. Primary antibodies anti-NTPDase1, anti-NTPDase2, anti-NTPDase3, and anti-ecto-5'-nucleotidase were developed in the Centre de Recherche en Rhumatologie et Immunologie, University Laval, Québec, Canada. Alexa Fluor 488-labeled anti-rabbit, Alexa Fluor 568-labeled anti-mouse, Alexa Fluor 653-labeled anti-goat and the fluorescent calcium indicator Fluo-4NW were supplied by Molecular Probes (Invitrogen, Carlsbad, CA). Dimethylsulphoxide (DMSO) was obtained from Merck (Frankfurt, Germany). Tissue culture plates: 96-well plates were purchased from Corning, Lowell, MA; FluoroDish plates for confocal microscopy were from World Precision Instruments (Hitchin, Hertfordshire, UK); chamber slides were from Nunc (Rochester, NY).

Presentation of data and statistical analysis

Results presented in this study are from bone marrow specimens obtained from eighteen individual female patients (68±5 years old, *n*=18). For each experiment

and assay, 6–8 replicas were accomplished. The data are expressed as mean ± S.E.M. from an *n* number of patients. Data from different individuals were evaluated using one-way analysis of variance (ANOVA) and no significant differences in the pattern of cell behaviour were found. Statistical differences found between control and drug-treated cultures were determined by Bonferroni's method. *P*-values <0.05 were considered to represent significant differences.

RESULTS

Phenotypic cells characterization by flow cytometry

Primary cultures were maintained for 10–15 days until near confluence when adherent cells were enzymatically released. The resultant cell suspension was cultured in conditions that favour osteogenic differentiation. Plastic-adherent MSCs (first subculture) were allowed to grow for 7 days. All the experiments were performed in the first subculture, since previous results from our collaborators and independent groups showed that serial passage of bone marrow-MSCs result in the progressive loss of the osteoblast phenotype detected as decreases in ALP activity and mineralization potential from the 1st to the 2nd subculture (Coelho et al., 2000; Fernandes et al., 1997; Schmidt and Kulbe, 1993).

MSCs are thought to be multipotent cells. These cells, which are present in the adult bone marrow, can replicate as undifferentiated cells and have the potential to differentiate to lineages of mesenchymal tissues, including bone. First passage MSCs prepared in the present experimental conditions were used for immunophenotypic analysis by flow cytometry. These cells exhibited positive immunoreactivity against several surface molecules, including CD105 (SH2), CD29 (integrin β1), and CD117 (tyrosine protein kinase Kit) (Figure 5), which have been identified as markers of bone marrow-derived mesenchymal stromal cells (Baddoo et al., 2003; Boiret et al., 2005; Cognet and Minguell, 1999; Dennis et al., 2002; Gronthos et al., 2003; Pittenger et al., 1999). Conversely, cells isolated from the human bone marrow and cultured under the experimental conditions were negative for haematopoietic surface markers, like

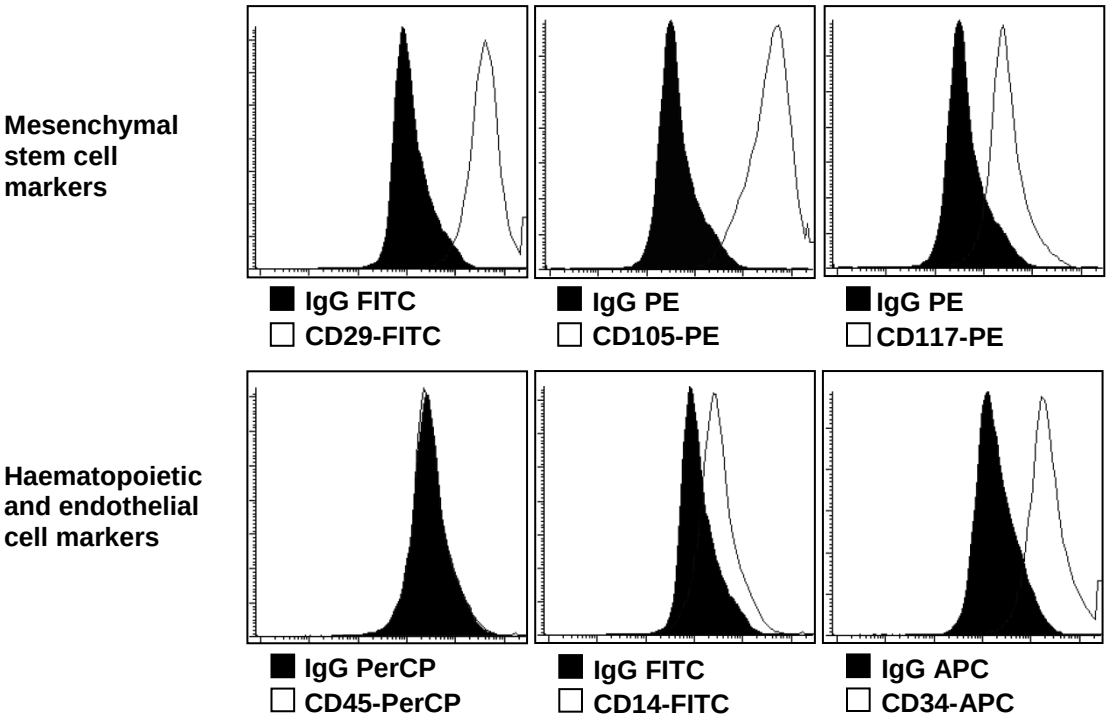

Figure 5. Flow cytometric analysis of surface markers of mesenchymal stem cells (MSCs) (CD29, CD105, and CD117) and haematopoietic and endothelial cells (CD45, CD14, and CD34) in plastic-adherent MSCs (first subculture) allowed to grow for 7 days in culture. Black histograms indicate isotype controls (fluorochrome-conjugates or secondary conjugated antibodies alone), white histograms show surface antigen expression level. Staining and stem cell markers have been chosen in accordance with the minimal criteria for defining multipotent mesenchymal stromal cells by the International Society for Cellular Therapy (Dominici et al., 2006). Cells were first identified based on their flow cytometric characteristics—high sideward (SSC) and forward (FSC) light scatter profile (total cell suspension). These cells were then gated on the basis of sideward scatter distribution, and they were analyzed for the expression of certain surface antigens recognized by the antibodies employed, namely anti-CD29 labelled with fluorescein isothiocyanate (FITC) (clone TS2/I6), anti-CD105 (mouse) labeled with phycoerythrin (PE) (clone IG2), anti-CD117 (mouse) labelled with phycoerythrin (PE) (clone 95C3), anti-CD45 (mouse) labelled with peridinin-chlorophyllprotein (PerCP) (clone 2D1), anti-CD34 (mouse) labelled with allophycocyanin (APC) (clone 8G12), and anti-CD14 (mouse) labelled with fluorescein isothiocyanate (FITC) (clone MwP9). Results are in mean fluorescence intensities, expressed in arbitrary relative linear units.

CD14 and CD45, which has been extensively used as a good argument to distinguish bone marrow haematopoietic cells from MSCs (Baddoo et al., 2003; Pittenger et al., 1999). Flow cytometry analysis showed that a subpopulation of human bone marrow cells were moderately positive for CD34, an endothelial and haematopoietic cell marker. Overall, we consider that under the present experimental conditions first passage plastic-adherent human bone marrow cells are highly enriched in multipotent mesenchymal stromal cells.

Osteoprogenitor cells from postmenopausal women exhibit low ALP activity

Human MSCs (first subculture) were allowed to grow for 28 days in an osteoblast-inducing medium. Meanwhile, cell cultures were characterized for proliferation and osteogenic differentiation events at days 1, 4, 7, 14, 21, and 28. Results regarding cell viability/proliferation were measured by the MTT assay. Cultures grown in control conditions showed a gradual increase in cell proliferation until day 28 (Figure 6A). We found no significant ($P>0.05$) differences regarding the proliferation profile (MTT assay) of the cells from both age groups, i.e., reduction of the tetrazolium salt (MTT) was similar between cells obtained from bone marrow specimens of female patients whose ages were 58–83 years (postmenopausal group, $n=18$) and 14–40 years (control group, $n=4$), respectively (Figure 6A). Results concerning total protein content reflected similar information as that obtained from the MTT assay (data not shown). The enzymatic MTT assay is often considered inappropriate to compare viability/proliferation of cells that can exhibit differences in their metabolic activity, such as cells from younger versus postmenopausal groups of patients. We, therefore, decided to compare the results from the MTT assay with those where cellularity and proliferation throughout the culture period was evaluated by quantifying total DNA content per culture well. Doing this, we also found no significant differences ($P>0.05$) in cell growth profiles of distinct age groups (Figure 6C). As expected, significant ($P<0.05$) differences were observed regarding the osteogenic differentiation profile (given by ALP activity) between the two groups of patients. Cell cultures from the younger group (patients of 14–40 years old, $n=4$) exhibit higher ALP activity than the postmenopausal group (patients of 58–83 years old, $n=18$). The ALP activity in the younger group of patients reached a maximum between days 7 and 21, decreasing significantly thereafter (Figure 6B), whereas in the postmenopausal group of patients the ALP activity increased slowly, yet progressively, with the incubation time until day 28. The biochemical results were confirmed by data from histochemical ALP staining, where the activity of ALP was identified by a progressive increase in the brownish staining of the cultures from day 7 to day 21 (Figure 6D). The differences in ALP activity found between the two age groups do

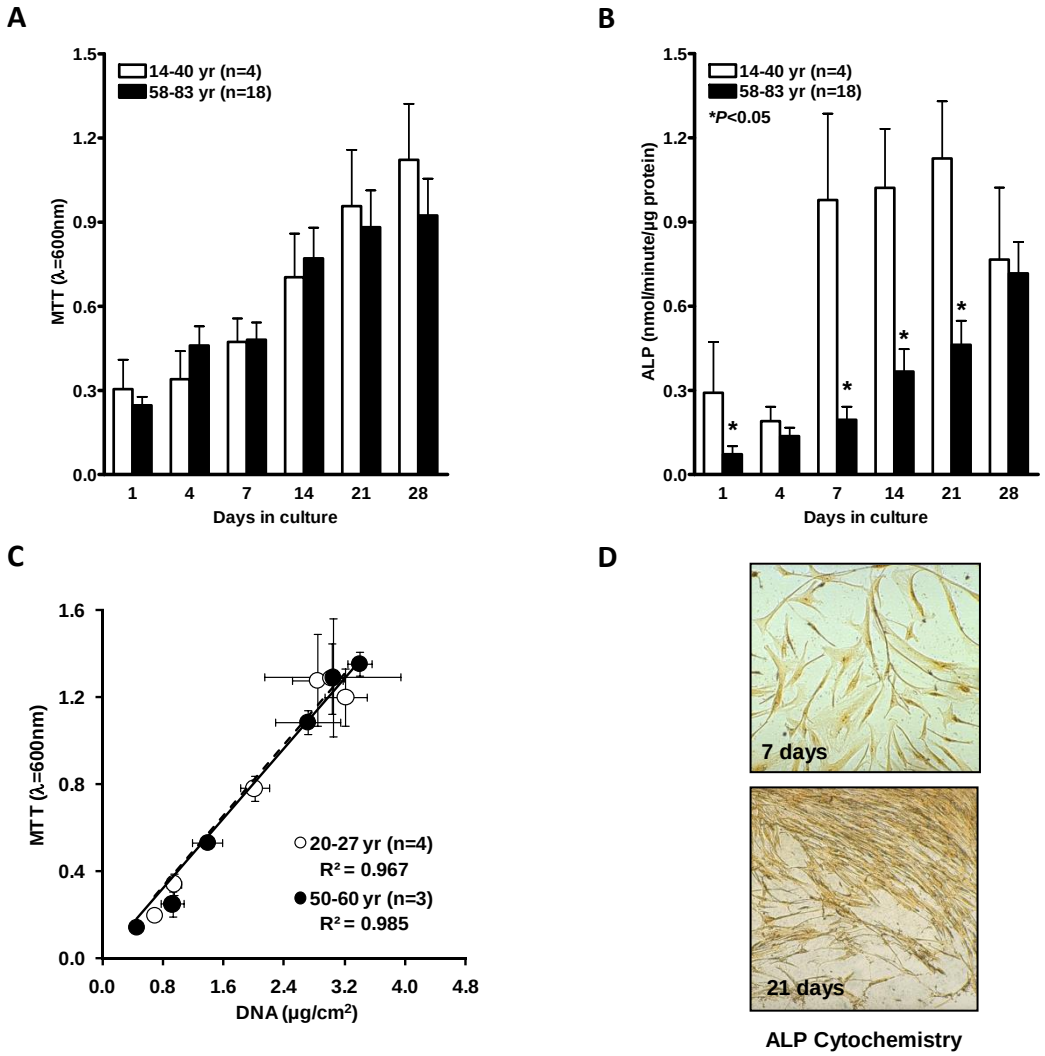

Figure 6. Proliferation and differentiation of mesenchymal stem cells (MSCs, first subculture) grown in an osteoblast inducing medium during 28 days. Bone marrow specimens were obtained from female patients with 58–83 years (postmenopausal group, *n*=18, black bars) and 14–40 years (control group, *n*=4, white bars) (see Materials and Methods section). Panel (A) represents cell viability/proliferation measured by the MTT assay; results are expressed as absorbance determination at 600nm per cm^2 at certain time points. Panel (B) represents the activity of alkaline phosphatase (ALP) in cell lysates; results are expressed in nanomoles of *p*-nitrophenol produced per min per µg of total protein at certain time points. Panel (C) shows that data from the MTT assay correlates positively with the results from DNA quantification measuring cell proliferation in two groups of female patients ageing 20–27 years old (control group, *n*=4) and 50–63 years old (postmenopausal group, *n*=3); DNA quantification is expressed as µg of DNA per cm^2 at certain time points. No significant ($P > 0.05$) differences were found between the two age groups. Six to eight replicas were performed for each individual experiment. The vertical and horizontal bars represent S.E.M. *$P < 0.05$ represent significant differences between the two age groups for certain time points. Panel (D) represents the presence of ALP at culture days 7 and 21 by cytochemistry (brown to black staining, according to the enzyme content). Images are representative of three independent experiments. Zoom 100X.

not reflect differences in cell proliferation since cell growth rate profiles of patients

with different ages were virtually similar (see Figure 6C). While we do not know the contribution of the smaller CD34 positive haematopoietic cell subpopulation to quantitative ALP measurements as compared to the expanded multipotent mesenchymal stromal cell population undergoing osteogenic progression in our cultures, it is interesting to note that haematopoietic cells rarely express ALP activity (Borgen et al., 1998).

Effect of uracil nucleotides and their stable analogues in the proliferation and osteogenic differentiation of human primary MSCs

First passage human primary MSCs were cultured for 21 days in conditions that favour osteogenic differentiation. We tested UTP (100 µM), UDP (100 µM), and their enzymatically stable derivatives, UTPγS (100 µM) and PSB 0474 (10 µM), respectively (Figures 7 and 8). UTP is one of the most potent $P2Y_2$ receptor agonists, but the nucleotide is also very active at $P2Y_4$ receptors. UTP derivatives containing the sulphur group, like UTPγS, are relatively potent $P2Y_2$ receptor agonists, but are only moderately selective or non-selective versus $P2Y_4$ receptors (see *e.g.*, El-Tayeb et al., 2006). In contrast, UDP analogues containing large phenacyl substituents at N3, like PSB 0474, are potent and selective $P2Y_6$ receptor agonists with no affinity for other uracil-sensitive P2Y receptors in the low micromolar concentration range. Continuous application of the agonists to the culture medium was devoid of effect on the proliferation of human primary MSCs measured by the MTT assay (data not shown).

The results concerning ALP activity are shown in Figures 7A and 8. Continuous treatment of the cells with UTP (100 µM) and UDP (100 µM) significantly ($P<0.05$) increased ALP activity during the first week as compared to controls tested in the absence of uracil nucleotides (Figure 7A). The effect of both UTP (100 µM) and UDP (100 µM) decreased progressively thereafter; at day 21, the activity of ALP was about the same in the presence of UTP (100 µM) and UDP (100 µM), as compared to control cultures (Figure 7A). The stable UTP analogue,

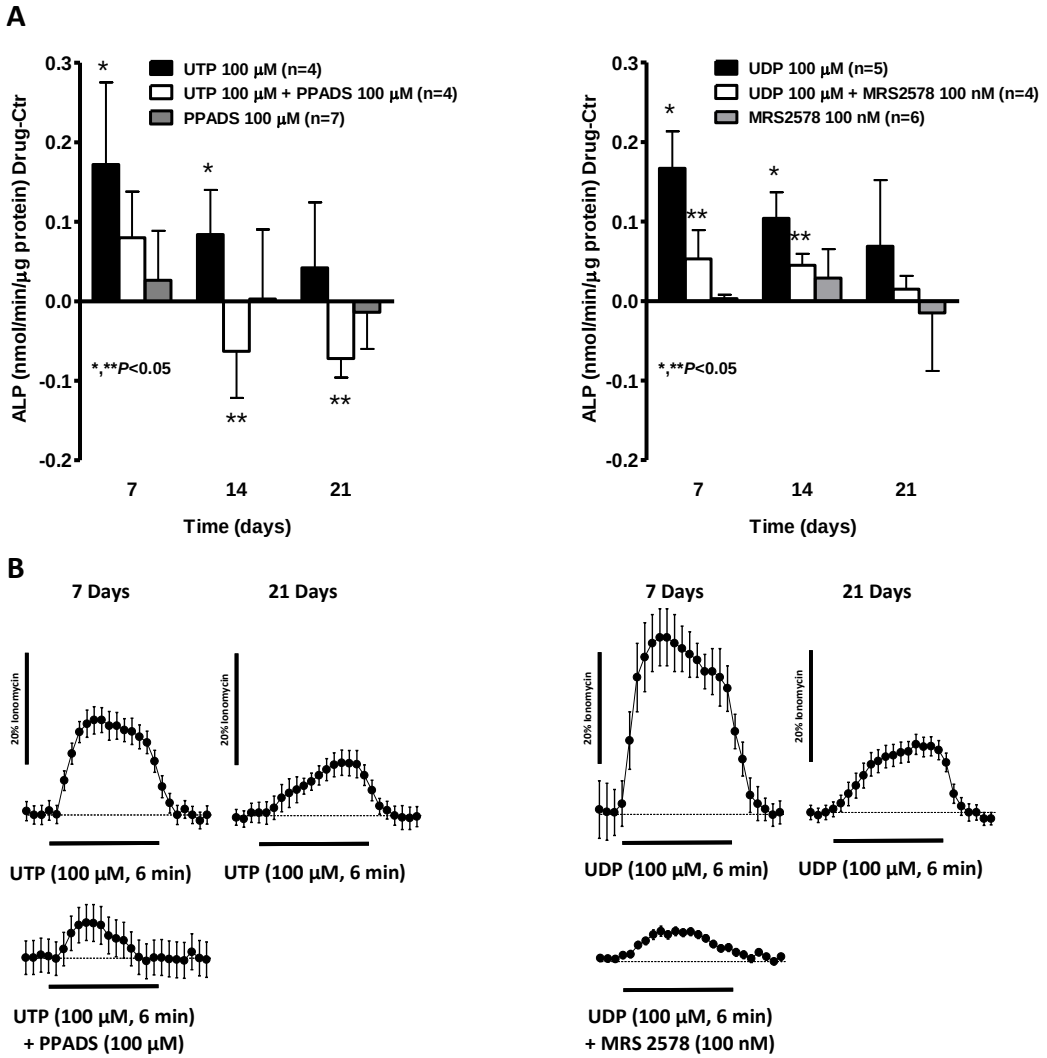

Figure 7. Panel (A), represented is the variation of alkaline phosphatase (ALP) activity of human primary bone marrow-derived mesenchymal stem cells (MSCs, first subculture) exposed continuously (during 21 days) to UTP (100 µM) and UDP (100 µM) in the absence and in the presence of PPADS (100 µM) and MRS 2578 (100 nM), respectively. For comparison purposes PPADS (100 µM) and MRS 2578 (100 nM) were also tested alone. The ordinates represent changes in the activity of ALP as compared to control cultures grown in the absence of test drugs at the same time points (see Figure 6). Zero represents identity between the two values (drug vs. control); positive and negative values represent facilitation or inhibition of osteogenic cell differentiation relative to control data obtained at the same time points. Each column represents pooled data from an *n* number of individuals; 6–8 replicas were performed for each individual experiment. The vertical bars represent S.E.M. **P* < 0.05 represent significant differences from control values obtained in the absence of tested drugs; ***P* < 0.05 represent significant differences compared with the effect of the agonist in the presence of its corresponding antagonist (PPADS or MRS 2578). Panel (B), represented is the fluorescence intracellular Ca^{2+} oscillations in human primary MSCs at culture days 7 and 21 caused by UTP (100 µM) and UDP (100 µM) in the absence and in the presence of PPADS (100 µM) and MRS 2578 (100 nM), respectively. Cells were pre-incubated with the fluorescent calcium indicator, Fluo4-NW (2.5 µM, in PBS plus 2.5% pluronic acid), for 45 min at 37^0C.

Figure 7. *(Continued)* Changes in fluorescence were detected by laser-scanning confocal microscopy (Olympus FV1000, Tokyo, Japan) in the time-lapse mode. Intracellular Ca^{2+} transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). The black bars at the bottom of each graph indicate the period of drug exposure. Each point represents pooled data from: 64 cells (UTP 100 µM, four different individuals, day 7), 55 cells (UTP 100 µM, three different individuals, day 21), 37 cells (UTP 100 µM plus PPADS 100 µM, three different individuals, day 7), 82 cells (UDP 100 µM, four different individuals, day 7; UDP 100 µM, three different individuals, day 21) and 72 cells (UDP 100 µM plus MRS 2578 100 nM, four different individuals, day 7). The vertical bars represent S.E.M.

UTPγS (100 µM), was devoid of effect on osteoblast cell differentiation measured by the increase of ALP activity in the cultures (Figure 8), suggesting that UTP has to be catabolised into UDP in order to facilitate differentiation of human osteoprogenitor cells. The selective $P2Y_6$ receptor agonist, PSB 0474, applied in the low micromolar (10 µM) concentration range, progressively increased ALP activity from the first week onwards reaching a maximum on culture day 21 (Figure 8). In order to know whether the effects of uracil nucleotides in ALP activity in human primary MSC cultures were mediated by subtype specific P2Y receptors, we tested their effects in the presence of PPADS (100 µM, a non-selective P2Y receptor antagonist) and MRS 2578 (100 nM), a selective $P2Y_6$ receptor antagonist displaying no activity at UTP-sensitive $P2Y_2$ and $P2Y_4$ receptors nor at ADP-activated $P2Y_1$ responses (Mamedova et al., 2004). PPADS (100 µM) attenuated the increase in ALP activity caused by UTP (100 µM) (Figure 7A). Blockade of $P2Y_6$ receptors with MRS 2578 (100 nM), significantly ($P<0.05$) attenuated the increases in ALP activity caused by both UDP (100 µM) (Figure 7A) and PSB 0474 (10 µM) (data not shown). When used alone, neither PPADS (100 µM, $n=7$) nor MRS 2578 (100 nM, $n=6$) changed cell proliferation and osteogenic differentiation as compared to controls (Figure 7A). These results indicate (1) that, on their own, PPADS (100 µM) and MRS 2578 (100 nM) are unable to change ALP activity in human primary MSCs cultures, and (2) that extracellular accumulation of uracil nucleotides in non-stressed cell cultures is probably not enough to activate selectively any of its receptors.

Uracil nucleotides promote increases in intracellular Ca^{2+} in human primary MSCs

Human primary MSCs cultured for 7 or 21 days in supplemented α-MEM medium were challenged with 100 μM UTP and UDP applied in the absence and in the presence of PPADS and MRS 2578, respectively. Both UTP and UDP

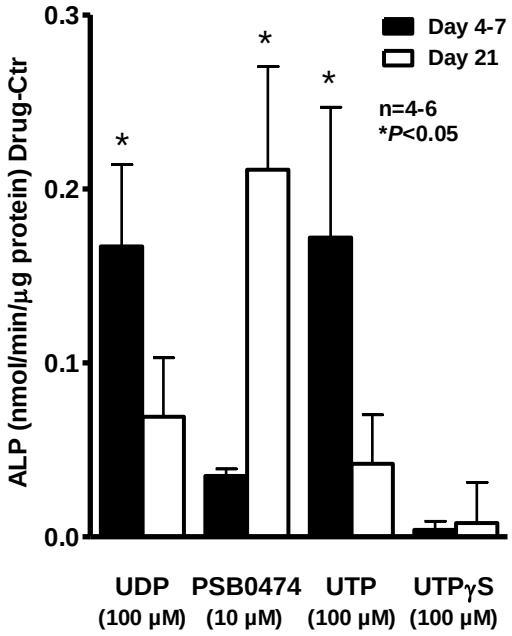

Figure 8. Variation of alkaline phosphatase (ALP) activity of human primary bone marrow-derived mesenchymal stem cells (MSCs, first subculture) exposed continuously during 7 or 21 days to UTP (100 μM), UDP (100 μM), and their stable analogues, respectively UTPγS (100 μM) and PSB 0474 (10 μM). The ordinates represent changes in the activity of ALP as compared to control cultures grown in the absence of test drugs at the same time points (see Figure 6). Zero represents identity between the two values (drug vs. control); positive and negative values represent facilitation or inhibition of osteogenic cell differentiation relative to control data obtained at the same time points. Each column represents pooled data from an *n* number of individuals; 4–6 replicas were performed for each individual experiment. The vertical bars represent S.E.M. **P* < 0.05 represent significant differences from control values obtained in the absence of tested drugs.

triggered increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$), measured with the intracellular fluorophore, Fluo-4NW, using a laser-scanning confocal microscope (Figure 7B). At culture day 7, we observed sustained responses that typically peaked 2 min after application of uracil nucleotides and decayed back to the baseline after washout of the drug. UDP was more potent than UTP, i.e., when the cells were challenged with UTP (100 μM) and UDP (100 μM) single-cell fluorescence $[Ca^{2+}]_i$

signals increased on average 21±8% (*n*=64 cells from four patients) and 40±3% (*n*=82 cells from four patients) of the maximal response produced by the calcium ionophore, ionomycin (5 μM), under the same experimental conditions (Figure 7B). Pre-incubation with PPADS (100 μM) significantly (*P*<0.05) attenuated $[Ca^{2+}]_i$ rises caused by UTP (100 μM, *n*=37 cells from three patients) (Figure 7B). Selective blockade of $P2Y_6$ receptors with MRS 2578 (100 nM) prevented the $[Ca^{2+}]_i$ responses triggered by UDP (100 μM, *n*=72 cells from four patients) (Figure 7B). Intracellular Ca^{2+} responses strikingly decreased with time in culture. For instance, ionomycin (5 μM)-calibrated single-cell $[Ca^{2+}]_i$ rises caused by UTP (100 μM) and UDP (100 μM) were about 3–4-fold greater in 7-day cultures than in 21-day cultures (Figure 7B). This contrast with the twofold increase in viable cells observed between days 7 and 21, as estimated by the MTT assay (see Figure 6). Consistently, more differentiated cells were less responsive to stimulation by uracil nucleotides, with only small increases in fluorescence $[Ca^{2+}]_i$ signals and ALP activity (Figure 7) being noted in 21-day cultures. In contrast to the pattern of fluorescence $[Ca^{2+}]_i$ signals observed in 7-day cultures, perfusion of the cells with uracil nucleotides caused a slow, yet progressive, rise in intracellular Ca^{2+} fluorescence which did not reach a peak until drug washout (Figure 7B).

Detection of $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor subtypes in postmenopausal primary MSCs by immunofluorescence

For immunocytochemistry, human primary MSCs were allowed to grow in chamber slides for 7 and 21 days in supplemented α-MEM. Under the present experimental conditions, osteogenic differentiation of human primary MSCs was also inferred by the increase in the immunoreactivity against type I collagen (85–90% of organic matrix) and the noncollagenous bone matrix protein, osteocalcin, comparing cells at culture days 7 and 21 (Figure 9).

Although the presence of the three subtypes of uracil sensitive P2Y receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) was confirmed in human primary MSCs by immunocytochemistry, staining changed significantly in intensity with time in culture (Figure 9). Immunoreactivity for $P2Y_2$ and $P2Y_4$ receptor proteins increased

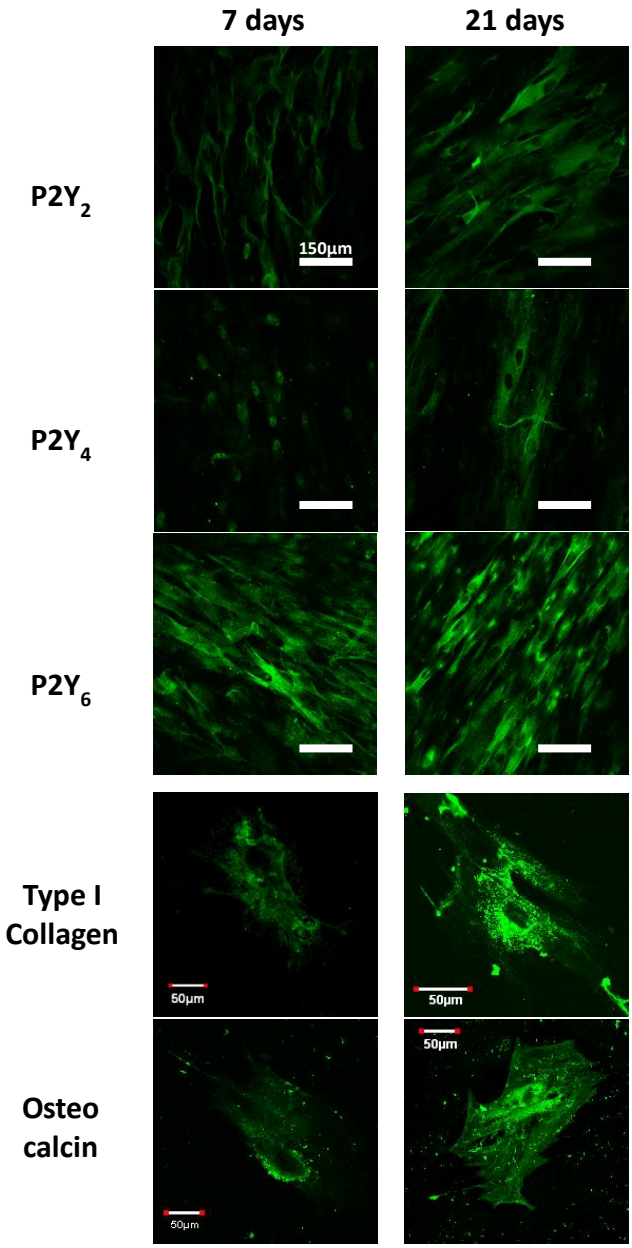

Figure 9. Immunocytochemical detection of uracil-sensitive P2Y receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$), type I collagen and osteocalcin on human primary bone marrow-derived mesenchymal stem cells (MSCs). Shown is the time-related immunoreactivity fluorescence detection by confocal microscopy of human MSCs (first subculture) allowed to grow for 7 and 21 days in an osteoblast-inducing medium. Cells grown in eight-well chamber slides were processed for immunocytochemistry in parallel and were visualized keeping unaltered the settings of the confocal microscope throughout the procedure. For further details on immunofluorescence labelling see Materials and Methods section.

significantly with time, whereas the $P2Y_6$ receptor protein expression remained fairly constant as cultures progressed. The immunostaining pattern for uracil-

sensitive P2Y receptors with time in postmenopausal primary MSCs was slightly different from that predicted in rat primary osteoblasts (Orriss et al., 2006). These authors showed that mature rat osteoblasts preferentially express the ATP- and UTP-sensitive $P2Y_2$ receptor and to a lesser extent $P2Y_4$ (also ATP/UTP selective) and $P2Y_6$ (UDP-sensitive) receptors.

ATP increases intracellular Ca^{2+} but decreases osteogenic differentiation of postmenopausal primary MSCs

For comparison, we also tested the effect of ATP (100 µM) on intracellular Ca^{2+} accumulation and osteogenic differentiation (measured by ALP activity) throughout MSC cultures life span in similar experimental conditions as those used for uracil nucleotides (Figure 10). Contrasting with the effect of uracil nucleotides on osteogenic differentiation (see above), continuous ATP (100 µM) application progressively decreased ALP activity from the first week onwards reaching a maximum on culture day 21 (Figure 10A). Acute superfusion of 7-day MSC cultures with ATP (100 µM) elicited a fast $[Ca^{2+}]_i$ rise, which typically peaked 40 sec after nucleotide addition and decayed back almost to baseline within 1–2 min; a second progressive, but of much lower amplitude, ATP-induced rise in intracellular Ca^{2+} was observed following the initial high-magnitude $[Ca^{2+}]_i$ transient (Figure 10B). Intracellular Ca^{2+} responses induced by ATP (100 µM) decreased with time in culture; for example, the late response to ATP almost disappeared at culture day 21 as compared to that observed in 7-day cultures (Figure 10B). Likewise, fast $[Ca^{2+}]_i$ transients calibrated by ionomycin (5 µM) were of smaller magnitude in 21-day cultures than in 7-day cultures. It is worth noting that ATP-induced fast $[Ca^{2+}]_i$ rises undergo rapid desensitization, i.e., application of a second ATP (100 µM) pulse following a washout period of at least 6 min elicited a significantly ($P<0.05$) smaller intracellular Ca^{2+} response than that observed with the first pulse (Figure 10B, inserts). Consecutive UTP applications induced $[Ca^{2+}]_i$ transients which were both of a similar magnitude, thus indicating that uracil nucleotide-sensitive P2 purinoceptors are not prone to desensitization.

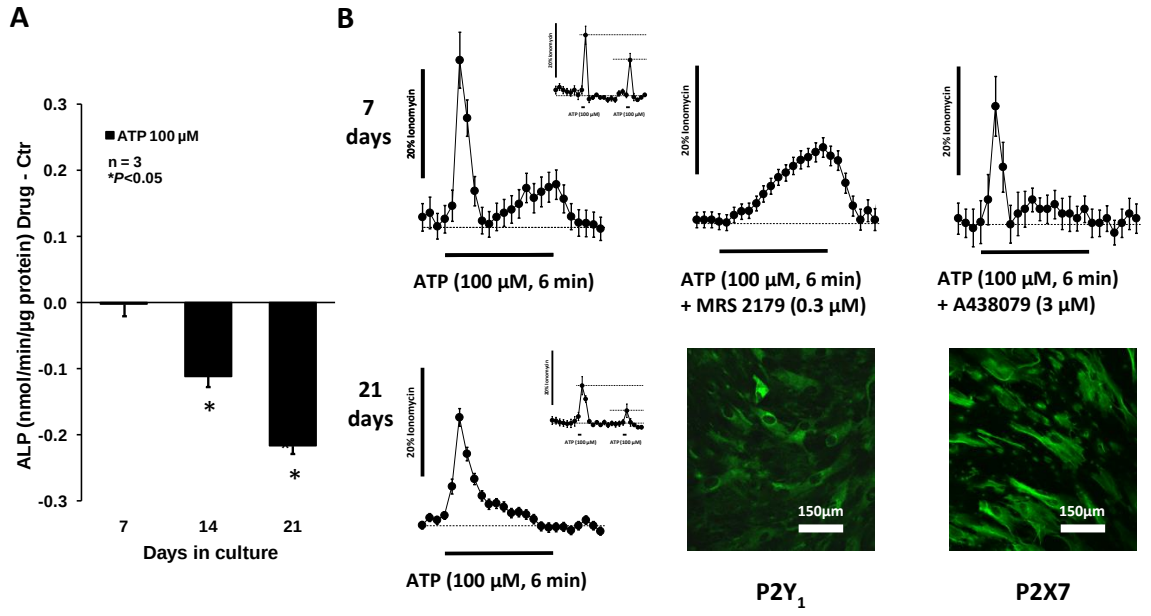

Figure 10. Effects of ATP (100 µM) on intracellular Ca^{2+} accumulation and osteogenic differentiation (measured by ALP activity) of human primary bone marrow-derived mesenchymal stem cells (MSCs) kept in culture during 21 days. Panel (A), represented is the variation of alkaline phosphatase (ALP) activity of human MSCs (first subculture) exposed continuously to ATP (100 µM) during 21 days as compared to control cultures grown in its absence at the same time points (see Figure 6). Zero represents identity between the two values (drug vs. control); positive and negative values represent facilitation or inhibition of osteogenic cell differentiation relative to control data obtained at the same time points. Each column represents pooled data from an *n* number of individuals; 6–8 replicas were performed for each individual experiment. The vertical bars represent S.E.M. *P < 0.05 represent significant differences from control values obtained in the absence of ATP (100 µM). Panel (B), represented is the fluorescence intracellular Ca^{2+} oscillations in human MSCs at culture days 7 and 21 caused by ATP (100 µM) in the absence and in the presence of selective $P2Y_1$ and P2X7 receptor antagonists, respectively MRS 2179 (0.3 µM) and A438079 (3 µM). Cells were pre-incubated with the fluorescent calcium indicator, Fluo4-NW (2.5 µM, in PBS plus 2.5% pluronic acid), for 45 min at 37^0C. Changes in fluorescence were detected in the time-lapse mode by laser-scanning confocal microscopy (Olympus FV1000, Tokyo, Japan). Intracellular Ca^{2+} transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Each point represents pooled data from: 78 cells (ATP 100 µM, three different individuals, day 7), 58 cells (ATP 100 µM, three different individuals, day 21), 64 cells (ATP 100 µM plus MRS 2179 0.3 µM, three different individuals, day 7) and 47 cells (ATP 100 µM plus A438079 3 µM, three different individuals, day 7). The vertical bars represent S.E.M. $[Ca^{2+}]_i$ transients resulting from two consecutive ATP (100 µM, for 1 min) applications 6 min apart from each other to human MSCs cultures (7 and 21 days), are also shown for comparison purposes (figure inserts). The black bars at the bottom of each graph indicate the period of drugs exposure. Human MSCs exhibit immunoreactivity against $P2Y_1$ and P2X7 receptors at culture day 7. Images obtained under the confocal microscope are representative of three independent experiments. Scale bar is 150 µm.

Recent studies have demonstrated that P2 receptor expression in osteoblasts is strongly differentiation-dependent (Orriss et al., 2006). Besides uracil activated receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) (see above), human osteoblast-like cells

express $P2Y_1$ receptors as indicated by RT-PCR (Maier et al., 1997) and immunolabeling (see Figure 10B) studies. Activation of $P2Y_1$ receptor is thought to modulate osteoblast responses to systemic factors such as parathyroid hormone via intracellular Ca^{2+} mobilization (Bowler et al., 1999; Buckley et al., 2001). Controversy however exists on the role of ATP activated P2X receptors, namely the P2X7 receptor, on human osteoblasts (Orriss et al., 2010; see also for a detailed review on the P2X7 receptor, Grol et al., 2009). Early reports suggested that P2X7 receptor activation caused enhanced osteoblast apoptosis (Gartland et al., 2001). The P2X7 receptor is also thought to mediate the ERK1/2 activation caused by fluid shear stress in osteoblast-like cells, via increased $[Ca^{2+}]_i$ and protein kinase C activation (Liu et al., 2008b). These findings prompted us to investigate the expression of $P2Y_1$ and P2X7 receptors on postmenopausal osteoprogenitor cells in culture by immunofluorescence confocal microscopy to see whether they could be implicated in ATP-induced intracellular Ca^{2+} responses and decreased osteogenic cells differentiation.

Figure 10B shows that postmenopausal MSCs in culture exhibit immunoreactivity against $P2Y_1$ and P2X7 receptors. As previously demonstrated by RT-PCR analysis in rat primary osteoblasts (Orriss et al., 2006), $P2Y_1$ and P2X7 receptors immunoreactivity was detected at early time points (culture day 7), but it declined thereafter (data not shown). These findings are in keeping with our results showing that ATP-induced intracellular Ca^{2+} responses decreased significantly at culture day 21 as compared to those observed in 7-day cultures (Figure 10B). Pre-incubation of 7-day cultures with the subtype selective $P2Y_1$ receptor antagonist, MRS 2179 (0.3 μM), totally abolished the fast $[Ca^{2+}]_i$ transient induced by ATP (100 μM), while keeping the late component of $[Ca^{2+}]_i$ rise. Conversely, the selective P2X7 receptor antagonist, A438079 (3 μM), significantly attenuated the late $[Ca^{2+}]_i$ response induced by ATP (100 μM) without much affecting the fast component. These results suggest that, in contrast to uracil nucleotides, ATP (100 μM) or its metabolite ADP operate biphasic $[Ca^{2+}]_i$ responses in postmenopausal MSCs in culture, which are respectively mediated by fast desensitizing $P2Y_1$ receptors and by slow activating (non-desensitizing)

P2X7 receptors. More recent studies from Dixon's group suggested that P2X7 stimulation leads to increased membrane blebbing and bone formation (Panupinthu et al., 2007, 2008). Thus, the potential role of the P2X7 receptor in osteoblasts deserves further clarification and so does the involvement of other P2X receptor subtypes which may be also expressed in human osteoblast-like cells.

Pattern of the extracellular catabolism of ATP and uracil nucleotides (UTP and UDP) in postmenopausal primary MSC cultures by HPLC

Figure 11 illustrates the time course of the extracellular catabolism of ATP and uracil nucleotides (UTP and UDP) in intact postmenopausal primary MSCs in culture. ATP (100 μM) was catabolised with a half-degradation time of 24±2 min (*n*=9 observations from three individuals) and 12±1 min (*n*=9 observations from three individuals) at culture days 7 and 21, respectively (Figure 11A, B, see also Table 4). Because enzymatic activity is usually represented as a function of the total protein content, yet in osteoblast differentiating cultures type I collagen accounts to 85–90% of the organic matrix, we decided to normalize the ecto-nucleotidase activity by the amount of viable cells given by the MTT assay (Table 4). Normalization of the activity of the enzyme hydrolysing ATP to the total protein content or to the amount of viable cells demonstrates that speed up of ATP catabolism as cultures progressed is due predominantly to the concomitant increase in the number of cells, since the net enzymatic activity decreased by 25–50%.

Postmenopausal primary MSCs allowed to grow for 7 days hydrolysed ATP to adenosine with a very modest appearance of ADP and AMP (Figure 11A). In contrast with the majority of tissue systems, MSCs exhibit low level of ecto-adenosine deaminase activity (Costa et al., 2011), so that adenosine progressively accumulates in 7-day cultures to a maximum of 38.3±3.2 μM 30 min after ATP (100 μM) application with only negligible (<1 μM) inosine and hypoxanthine formation. At 21-day cell cultures, ADP accumulation in the medium transiently

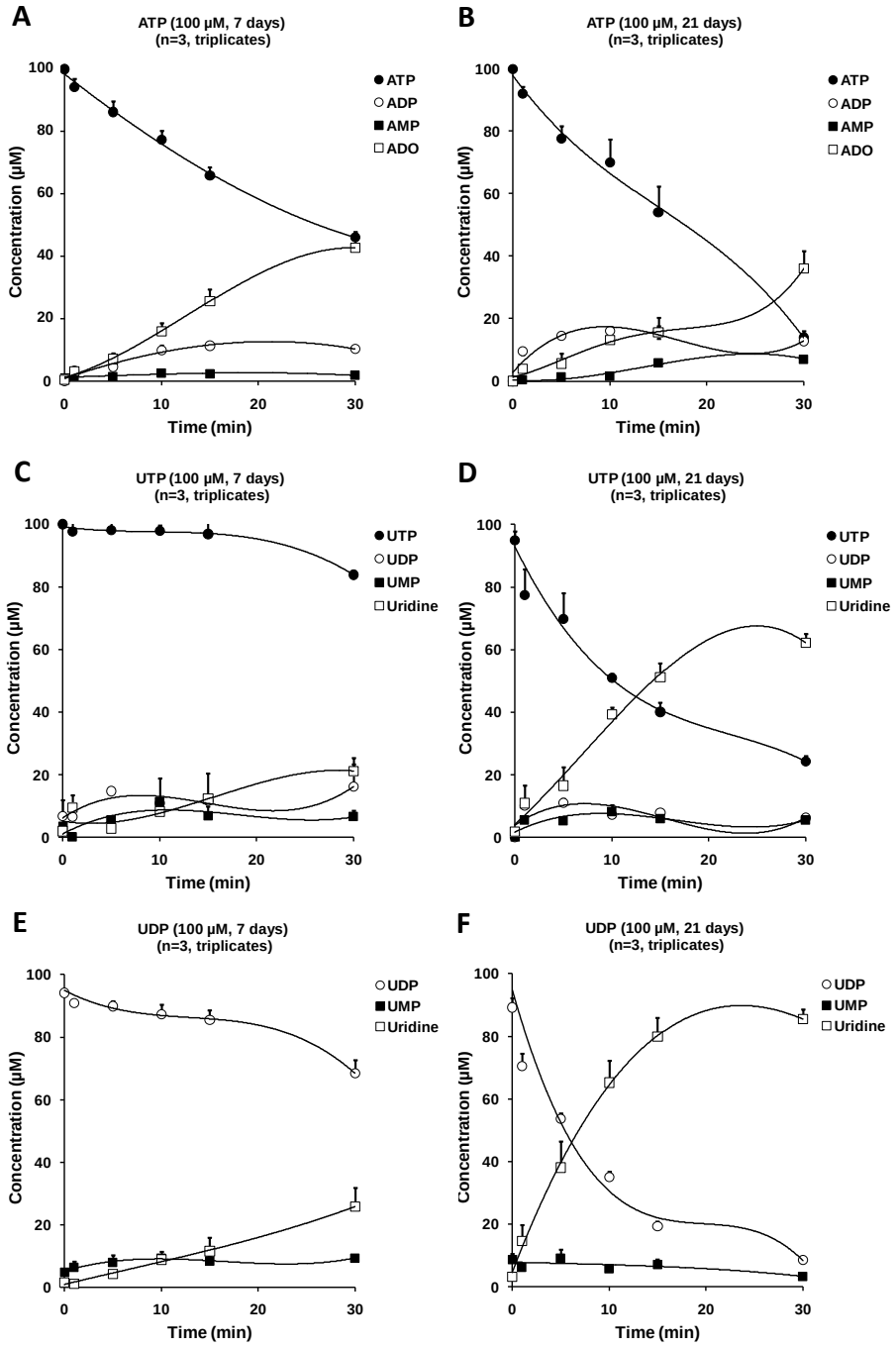

Figure 11. Time course of extracellular ATP (A and B), UTP (C and D), and UDP (E and F) metabolism in human primary bone marrow-derived mesenchymal stem cells (MSCs) in culture at days 7 (A, C, and E) and 21 (B, D, and F). Nucleotides (100 µM) were added to the culture medium at time zero. Samples (75 µl) were collected from each well at indicated times in the abscissa. Each collected sample was analysed by HPLC to separate and quantify ATP or UTP (filled circles), ADP or UDP (open circles), AMP or UMP (filled squares), and adenosine (ADO) or uridine (open squares). Each point represents pooled data from three individuals; three replicas were performed in each individual experiment. The vertical bars represent S.E.M. and are shown when they exceed the symbols in size.

increased to a maximum of 16.0±0.5 μM 10 min after ATP (100 μM) application (Figure 11B). Formation of adenosine from dephosphorylation of AMP by ecto-5'-nucleotidase (CD73 membrane-cell maker, EC 3.1.3.5) was moderately delayed as compared to 7-day cultures; adenosine reached a maximal concentration of 36.0±5.6 μM 30 min after ATP (100 μM) application to human MSCs cultured for 21 days. Given the linearity of the semi-logarithmic progress curves obtained by polynomial fitting of the catabolism of ATP and AMP, the analysis of the corresponding half-degradation time values suggests that the extracellular catabolism of ATP into AMP through ecto-nucleotidases is the rate-limiting step to generate adenosine from exogenously added adenine nucleotides in MSC cultures (*cf*. Costa et al., 2011).

Extracellular UTP (100 μM) was catabolised with a half degradation time of 143±29 min (*n*=9 observations from three individuals) and 12±1 min (*n*=9 observations from three individuals) at culture days 7 and 21, respectively (Figure 11C, D). The UTP metabolites detected in the bath were UDP, UMP, and uridine, whose concentrations increased with time in human MSC cultures (Figure 11C, D). UDP transiently accumulated to maximal concentrations of 14.7±1.3 μM and 11.1±1.2 μM 5 min after UTP (100 μM) application to 7- and 21-day cultures, respectively. Uridine progressively accumulated in human MSC cultures to maximal concentrations of 21.1±2.3 μM and 62.2±2.8 μM 30 min after UTP (100 μM) application at days 7 and 21, respectively. The net enzymatic inactivation of UTP normalized to the total protein content or to the amount of viable cells increased by 6–10-fold as cultures progressed from day 7 to 21, respectively (Table 4). Given that the number of viable cells increased only by about twofold in the same period of time in culture, the results suggest that more differentiated human MSCs exhibit higher ecto-nucleotidase activity implicated in the inactivation of extracellular UTP. In these circumstances, the kinetics of inactivation of UTP and ATP become comparable yet differences may be noted in the profile of products formation (Figure 11).

The progress curves of UDP (100 μM) disappearance in postmenopausal primary MSC cultures at days 7 and 21 are represented in Figure 11 E, F,

Table 4. Ectonucleotidase activity and half-degradation time values of ATP, UTP and UDP on human primary bone marrow-derived mesenchymal stem cells (MSCs) during osteogenic differentiation.

	Day 7			Day 21		
	t½ (min)	pmol/µg prot. /15 min	pmol/MTT abs. /15 min	*t½* (min)	pmol/µg prot. /15 min	pmol/MTT abs. /15 min
ATP	24±2	173±32	6488±1203	12±1*	82±15*	4871±899
UTP	143±29	16±14	576±492	12±1**	85±5**	5042±254**
UDP	69±16	60±14	2255±492	6±1**	114±2**	6783±122**

ATP, UTP and UDP (100 µM) were added to culture media of human primary MSCs at days 7 and 21. Samples (75 µl) were collected from each well at times 1, 5, 10, 15, and 30 min after application of the substrate. Each collected sample was analysed by HPLC to separate and quantify ATP and/or UTP, ADP and/or UDP, AMP and/or UMP, and adenosine and/or uridine (see Figure 11). Enzymatic activity is usually presented as a function of the total protein content (day 7: 17.5±2.6 µg/cm^2 vs. day 21: 52.5±10.6 µg/cm^2, n=14). Yet, in osteoblast differentiating cultures type I collagen accounts to 85–90% of the organic matrix, thus normalization of the ectonucleotidase activity was also done by the amount of viable cells given by the MTT assay (day 7: 0.480±0.024 A/cm^2 vs. day 21: 0.892±0.049 A/cm^2, n=20). Values are means SEM from three individuals; three replicas were performed in each individual experiment.
*P<0.05 represents significant differences when compared with day 7.
**P<0.01 represents significant differences when compared with day 7.

respectively. The results show that extracellular UDP (100 µM) is slowly inactivated in less differentiated cultures; the rate UDP catabolism speeds up (2–3-fold) significantly as cultures progressed to more differentiate status. That is, UDP (100 µM) was catabolised with a half-degradation time of 69±16 min (n=9 observations from three individuals) and 6±1 min (n=9 observations from three individuals) at culture days 7 and 21, respectively (Figure 11E, F). The UDP metabolites detected in the bath were UMP and uridine. Thirty minutes after UDP (100 µM) application, the concentration of uridine in the culture medium was about 25.8±6.0 µM and 85.5±3.2 µM in samples collected from cell cultures at days 7 and 21, respectively. At culture day 21, the enzymatic inactivation rate of UDP normalized to the total protein content or to the amount of viable cells was the strongest of all nucleotides tested, whereas it had only intermediate values in 7-day cultures (Table 4). This might explain why UDP does not easily accumulate in

more mature MSC cultures when UTP (100 μM) was used as a substrate (Figure 11D).

The presence of a saturating concentration (10 mM) of β-glycerophosphate in the culture medium did not alter the degradation kinetics of ATP and uracil nucleotides (UTP and UDP), suggesting that the contribution of non-specific phosphatases (*e.g.*, ALP, EC 3.1.3.1) to the extracellular catabolism of nucleotides applied in a 100-μM concentration is negligible (data not shown).

Detection of NTPDase1, -2, -3, and ecto-5'-nucleotidase in postmenopausal primary MSCs by immunofluorescence

Comparing the kinetics of adenine and uracil nucleotides hydrolysis suggests that the former are much better substrates than uracil nucleotides for enzymes of the ecto-nucleotidase family putatively expressed in less differentiated (7-day) human MSC cultures. Reversion of this enzymatic pattern that plays a major role in tri- and diphosphonucleoside hydrolysis may be a hallmark of osteogenic differentiation of human MSCs in culture and has the potential to fine-tuning regulate P2 receptors signalling in the human bone.

Human primary MSCs were allowed to grow in chamber slides for 7 and 21 days in supplemented α-MEM before immunolabeling with specific primary antibodies directed against human NTPDase1, -2 and -3, and ecto-5´-nucleotidase. Fluorescence immunoreactivity against ecto-NTPDase1, -2 and -3, and ecto-5'-nucleotidase in human primary MSCs shows a cytoplasmic/membrane-staining pattern typical for ectoenzymes, which increased in intensity with time (7<21 days) in culture (Figure 12). This is particularly evident for NTPDase1 and -3 (Figure 12A, C). Interestingly, immunoreactivity against NTPDase2 was almost unapparent at 7-day cultures (Figure 12Bi) and became stronger in more differentiated (21-day) cell cultures (Figure 12Bii). Human primary MSCs also exhibited positive immunoreactivity against CD73 (ecto-5'-nucleotidase) that is found to be expressed exclusively (>95%) in osteoprogenitors (see *e.g.*, Liu et al., 2009). Immunolabeling of CD73 molecules in postmenopausal

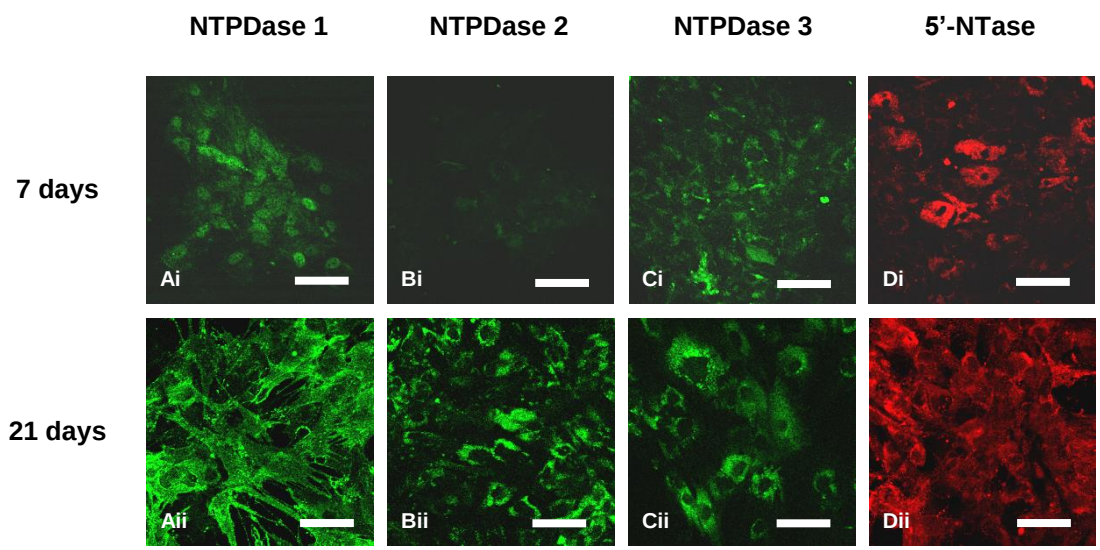

Figure 12. Immunocytochemical detection of NTPDase1, -2, and -3 and ecto-5'-nucleotidase on human primary bone marrow-derived mesenchymal stem cells (MSCs). Shown is the time-related immunoreactivity fluorescence detection by confocal microscopy of human MSCs (first subculture) allowed to grow for 7 (Ai–Di) and 21 (Aii–Dii) days in an osteoblast-inducing medium. Cells grown in eight-well chamber slides were processed for immunocytochemistry in parallel and were visualized keeping unaltered the settings of the confocal microscope throughout the procedure. For further details on immunofluorescence labelling see Materials and Methods section. Scale bar is 150 µm.

primary MSCs was demonstrated by both immunocytochemistry (Figure 12) and flow cytometry (data not shown).

DISCUSSION

The actions of extracellular adenine nucleotides on the basic functions of bone cells appear to be relatively clear-cut. ATP and ADP exert striking *in vitro* actions at concentrations in the low micromolar range to stimulate the formation and resorptive activity of osteoclasts, whereas ATP decrease ALP activity (see *e.g.*, Figure 10A) and inhibit mineralized bone formation by osteoblasts (Hoebertz et al., 2003). Identification of uracil nucleotides-sensitive $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors suggests that extracellular UTP/UDP have also important roles in regulating cellular function in a variety of tissues and organs. Few reports have, however, questioned the action of these compounds as autocrine/paracrine mediators in human bone physiology. In this study, we show that uracil

nucleotides, both UTP and UDP, are important enhancers of intracellular [Ca^{2+}] and osteogenic differentiation of human primary bone marrow-derived MSCs isolated from postmenopausal women, but their effects decrease in magnitude with time of the cells in culture. Paradoxically, these changes were notably accompanied by an increase in the expression of UTP-sensitive $P2Y_2$ and $P2Y_4$ receptors in differentiated osteoblasts (Figure 9; see also Orriss et al., 2006), whereas the expression of UDP-sensitive $P2Y_6$ receptor remained fairly constant along cells differentiation. Given that (1) UDP was more potent than UTP and that (2) the enzymatically stable UDP analogue, PSB 0474, enhanced ALP activity at all stages of MSC cultures whereas UTPγS was devoid of effect, it is reasonable to admit that the $P2Y_6$ receptor exerts a predominant role on osteogenic differentiation of bone marrow-derived MSCs from postmenopausal women. Another interesting finding from this study suggests that stimulation of $P2Y_6$ receptors by UDP originated from the catabolism of UTP in bone microenvironment may be balanced through the activity of type-specific membrane-bound NTPDases whose expression varies along MSCs maturation (see *e.g.*, Figure 12).

To our knowledge, this is the first study to investigate the role of uracil nucleotide-sensitive receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) on intracellular [Ca^{2+}] levels and osteogenic differentiation (measured as increases in ALP activity) of primary bone marrow-derived MSCs from postmenopausal female patients maintained in long-term cultures. Promotion of osteogenic differentiation of human MSCs by uracil nucleotides contrasts with the inhibitory action of ATP regarding ALP activity under similar experimental conditions (see Figure 10). The fact that UDP was more potent than UTP fits with the $P2Y_6$ being a dominant receptor type involved in osteogenic differentiation of human primary MSCs. Indeed, UDP was reported to be 100-fold more potent than UTP at the human recombinant $P2Y_6$ receptor (Communi et al., 1996). Selective activation of $P2Y_6$ receptors with PSB 0474 (El-Tayeb et al., 2006) mimicked the effects of both UTP and UDP, whereas the stable UTP analogue, UTPγS, was devoid of effect. Blockade of UDP-sensitive $P2Y_6$ receptors with MRS 2578 (Mamedova et al., 2004) prevented $[Ca^{2+}]_i$ rises

and osteogenic differentiation caused by UDP at all culture time points. Surprisingly, the effects of both UTP and UDP on intracellular [Ca^{2+}] and osteogenic differentiation became less evident as cultures progressed towards a more differentiated status. Immunofluorescence analysis revealed a strong differentiation dependence of P2 receptor expression in long-term human MSC cultures which was characterized by a striking increase in the levels of UTP-sensitive $P2Y_2$ and $P2Y_4$ receptors during the third week of culture, while the expression of the $P2Y_6$ receptor remained fairly constant along culture time. This receptor profile is compatible with that found by Orriss et al. (2006) in primary rat osteoblasts. As changes in subtype-specific P2 receptors expression could not afford for the decrease in the effects of uracil nucleotides on intracellular [Ca^{2+}] and osteogenic differentiation of human MSCs, we drew our attention towards the possibility that uracil nucleotides were increasingly broken down by membrane bound ecto-enzymes upon cells differentiation. The rate of extracellular UTP and UDP hydrolysis was 3 to 10-fold higher in more differentiated cell populations, which is consistent with the increase of NTPDase1, -2, and -3 immunolabeling on human MSCs with time in culture (Figure 12). Together, these data provide good evidence for significant up regulation of uracil nucleotides break down enzymes as osteogenic differentiation proceeds, which may rapidly terminate activation of the predominant ($P2Y_6$) receptor subtype.

The $P2Y_6$ receptor was cloned from a human cDNA library and was found to be expressed in transformed human osteoblast-like cells (Maier et al., 1997). In most instances, $P2Y_6$ receptors signal through pertussis toxin-insensitive G_q to activate PLCβ, leading to formation of IP3 and subsequent release of Ca^{2+} from intracellular stores (Ralevic & Burnstock, 1998). In agreement with those early findings (see also Orriss et al., 2006), we found strong pharmacological and immunocytochemical evidences for the expression of the $P2Y_6$ receptor on primary bone marrow-derived MSCs isolated from postmenopausal female patients, together with significant intracellular [Ca^{2+}] responses to UDP (the natural ligand selective for this receptor). Our data also show that stimulation of the $P2Y_6$ receptor promotes ALP activity at early culture stages, indicating the progression

of MSCs towards osteogenic differentiation. Unlike adenine nucleotides (*e.g.*, ATP), which downregulate ALP activity in human MSCs (see Figure 10), little else is known about the $P2Y_6$ receptor or the role that it might play in bone cell metabolism. Activation of $P2Y_6$ receptors may transiently activate NF-kB increasing survival of rabbit osteoclasts (Korcok et al., 2005), through the sequential activation of conventional protein kinase C (PKC) isoforms and IkB kinase leading to IkB degradation by the proteasome (Ye, 2001). Activation of $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors induces transient rises in intracellular $[Ca^{2+}]$, while only the $P2Y_6$ receptor couples to NF-kB activation, indicating that the rise in intracellular $[Ca^{2+}]$ alone is not sufficient to activate NF-kB (Korcok et al., 2005). It remains to be explored whether this pathway also operates in human osteoblast progenitors.

Besides the $P2Y_6$ receptor, UTP-sensitive $P2Y_2$ and $P2Y_4$ receptors have also been identified in osteoblast-like cell lines from both humans and rodents, but previous attempts failed to detect their expression in normal human osteoblasts (Maier et al., 1997; Orriss et al., 2006). Here, we demonstrate that the immunoreactivity against $P2Y_2$ and $P2Y_4$ receptors increased strongly between day 7 and 21 in human primary MSCs in culture. Immunohistochemical and quantitative real-time PCR studies revealed that $P2Y_2$ receptor expression increased as rat BMSC density increased (Ichikawa and Gemba, 2009). Paradoxically, responsiveness of these cells to UTP, which is the most potent agonist of $P2Y_2$ and $P2Y_4$ receptor subtypes, decreased in differentiated osteoblasts. Moreover, the hydrolysis-resistant UTP analogue, UTPγS, was without effect, suggesting that UTP has to be catabolised into UDP in order to facilitate differentiation of human osteoprogenitors predominantly through the activation of $P2Y_6$ receptors.

In agreement with early findings (reviewed by Hoebertz et al., 2003), we provided evidences confirming that ATP acts as a negative regulator of osteogenic differentiation of human postmenopausal bone marrow-derived MSCs in culture, whereas uracil nucleotides did the opposite. We also demonstrated that ATP operates biphasic $[Ca^{2+}]_i$ responses in osteoprogenitor cells, which are

respectively mediated by fast desensitizing $P2Y_1$ receptors and by slow activating (non-desensitizing) P2X7 receptors. Human osteoblast-like cells express $P2Y_1$ receptors as indicated by RT-PCR (Maier et al., 1997) and by immunolabeling (see Figure 10B) studies. Activation of $P2Y_1$ (and $P2Y_2$) receptors by extracellular ATP sensitizes osteoblast cells to $[Ca^{2+}]$ responses caused by parathyroid hormone (Bowler et al., 1999; Buckley et al., 2001). Synergism between ATP and parathyroid hormone activate bone remodelling at discrete sites of cell skeletal damage (Bowler et al., 2001). Conflicting results have been published on the expression and the potential role of the P2X7 receptor in osteoblasts. The P2X7 receptor was thought originally to be predominantly expressed in cells of the haematopoietic cell lineage, including macrophages and osteoclasts, which consist of a small percentage of the cells in our cultures (see Figure 5). However, recent studies have shown the expression of P2X7 receptors in a subpopulation of osteoblasts, both from human bone explants (see also Figure 10B), in the human osteosarcoma cell line SaOS-2, and in primary osteoblasts obtained from mouse calvarial cultures (reviewed by Hoebertz et al., 2003). Further, prolonged ATP stimulation induced pore formation in subpopulations of these osteoblast cultures as measured by ethidium bromide uptake. In the SaOS-2 cells, stimulation with the selective P2X7 receptor agonist BzATP not only induced pore formation, but also increased LDH release and initiated apoptotic processes in the cells, indicating that P2X7 receptors might be involved in the regulation of cell survival in osteoblasts. Interestingly, single nucleotide polymorphisms in the P2X7 receptor gene are associated with loss of lumbar spine bone mineral density and postmenopausal fracture risk in female patients (Ohlendorff et al., 2007).

The study of nucleotide receptors and their functions is complicated by the presence at the cell surface ectonucleotidases, which rapidly break down nucleotides into nucleosides (for a review, see Zimmermann, 2000). The broad functional diversity of the extracellular nucleotide-hydrolysing enzymes is not matched by a selective tissue distribution that might be expected if there were tissue-specific requirements in the extracellular nucleotide hydrolysis pathways. It is likely that enzymes belonging to different enzyme families are co-localized on

individual cell or tissue surfaces. Among the nucleotidases, NTPDase1, NTPDase2, NTPDase3, and NTPDase8, and two members of the ecto-nucleotide pyrophosphatases/ phosphodiesterases (E-NPP) family, NPP1 and NPP3, are located at the plasma membrane and hydrolyse extracellular nucleotides (Kukulski et al., 2005; Stefan et al., 2005; Zimmermann, 2000). Detailed studies using antibodies can differentiate between closely related enzyme species. Our findings showed that human primary MSCs express NTPDase1 and -3 at all differentiation time points (7 and 21 days in culture), whereas NTPDase2 immunoreactivity becomes evident only in more differentiated cells. Human NTPDase1 (also called CD39 or apyrase, EC 3.6.1.5) acts preferentially on adenine nucleotides (*Km*~20 μM) as compared with uridine nucleotides (*Km*~50 μM); this enzyme dephosphorylates ATP directly to AMP, removing one phosphate at a time with almost no release of intermediate ADP. The ADP hydrolysing capacity of NTPDase1 may even exceed the ATPase activity of the enzyme by up to 1.5–2 times. NTPDase2 (CD39L1, EC 3.6.1.3) conversely is a preferential nucleoside triphosphatase; this enzyme hydrolyses diphosphates 10–15 times less efficiently than nucleotide triphosphates, leading to minimal monophosphates accumulation (Matsuoka and Ohkubo, 2004). NTPDase3 (CD39L3 or HB6) and NTPDase8 (hepatic ATPDase) are described as functional intermediates between NTPDase1 and -2 (Kukulski et al., 2005). With the exception of NTPDase8, the apparent *Km* constants of human NTPDases obtained for adenine nucleotides as substrates are in the low micromolar range; higher *Km* values were obtained for the hydrolysis of uracil nucleotides. Interestingly, UTP is a much better substrate than UDP for NTPDase1 and -3 (Kukulski et al., 2005), which may influence the real potency of UTP on human MSCs in culture. Distinctively from adenine nucleotides, all membrane-bound NTPDases dephosphorylate UTP with a transient formation of UDP, thus favouring $P2Y_6$ receptor activation (Kukulski et al., 2005; see Figures 7 and 11). Expression of NTPDase2 in more differentiated (21 days in culture) human MSCs would be expected to terminate ATP and UTP activation of P2X7 and $P2Y_{2,4}$ receptors, respectively, which might contribute to up-regulate these receptors during osteogenic differentiation (Figure 9; see also Orriss et al., 2006).

Simultaneously, increased NTPDase2 activity could provide generation of ADP and UDP for stimulation of $P2Y_1$ and $P2Y_6$ receptors. However, the concomitant increase in the expression of NTPDase1 and -3 (see Figure 12) may efficiently hydrolyse ADP (data not shown) and UDP (see Table 4), thus precluding activation of receptors for these two nucleotides, $P2Y_1$ and $P2Y_6$, regulating intracellular [Ca^{2+}] accumulation. As for NPP1 and NPP3 (EC 3.6.1.9), they release nucleoside 5'-monophosphate plus Pi or PPi from a variety of nucleotides and nucleotide derivatives, but intriguingly, their phosphorylated product bind to NPPs with a higher affinity than their substrates do, and inhibit catalysis (Stefan et al., 2005). Osteoblasts express NPP1, NPP2, and NPP3 enzymes (Johnson et al., 2000; Orriss et al., 2007). The enzyme responsible for the final hydrolysis of nucleoside-5'-monophosphates to their respective nucleoside, ecto-5'-nucleotidase (CD73, EC 3.1.3.5), occurs in essentially all tissues. It is interesting to note that human MSCs exhibit high ecto-5'-nucleotidase activity and are immunoreactive against CD73, which is found to be expressed exclusively (>95%) in osteoprogenitors (Costa et al., 2011; Liu et al., 2009). Fast conversion of nucleoside-5'-monophosphates to their respective nucleosides and the kinetics of ATP/UTP inactivation through members of the E-NPP family in human MSC cultures may explain why the concentration of AMP/UMP in the extracellular milieu was kept at a low level while keeping high levels of PPi that contribute to inhibit bone mineralization (Hoebertz et al., 2003; Orriss et al., 2010). Altogether, our results suggest that NTPDases and NPP enzymes have the potential to exert a tight, distinct, and sophisticated regulation of subtype-specific P2 receptors signalling, and therefore affect intracellular [Ca^{2+}] and osteogenic differentiation of human primary MSCs maintained in long-term cultures. This is especially relevant considering the poverty of uracil metabolizing enzymes apart from the ectonucleotidases (Robson et al., 2006).

All cells contain high intracellular ATP concentrations (1–5 mM), as well as the capacity to release ATP following trauma. Given that intracellular ATP levels are 10–50 times higher than the other purine or pyrimidine nucleoside triphosphate species (including UTP) it is not surprising that at sites of tissue injury, wounding,

or fracture, micromolar concentrations of purine or pyrimidine nucleotides will be present to activate P2 receptors. Nevertheless, it is obvious that nucleotides must exist transiently in the bone microenvironment without cell damage to be physiologically relevant regulators of bone remodelling. There are two distinct nonlytic mechanisms by which nucleotides may be constitutively released from cells: (a) Exocytic release specifically concentrated within secretory granules or vesicles; and (b) release of cytosolic nucleotides via intrinsic plasma membrane channels or pores, which includes ABC transporters and connexin hemichannels controlled release (Bodin and Burnstock, 2001; Novak, 2003). Various agonists to P2 receptors can also initiate nucleotides release; in a positive feedback loop, UTP may act through $P2Y_2$ receptors to upregulate ATP release from primary human osteoblasts (Bowler et al., 1998). In addition, bone cells constitutively release ATP (and possibly UTP) upon mechanical stimulation, hypoxia, and hypo/hypertonic stress (reviewed by Bowler et al., 2001). The ability of various cell types (*e.g*., murine airway epithelial cells, human cardiomyocytes) to release UTP has been directly confirmed (Lazarowski et al., 1997, 2003). It is important to stress that the extracellular accumulation of nucleotides such as UTP, UDP, and ADP may not necessarily involve a requirement for their selective release from intracellular pools. Rather, these nucleotides may accumulate as a secondary consequence of extracellular metabolism (degradation or synthesis) of nucleotide precursors that are directly released in various physiological or pathological conditions (Burrell et al., 2005; Buxton et al., 2001; Joseph et al., 2004; Yegutkin et al., 2001, 2002). Thus, ADP or UDP may either be released directly or be generated via the extracellular hydrolysis of directly released ATP or UTP. It is also possible that extracellular UTP may be secondarily generated via an ecto-nucleoside diphosphokinase (NDPK)-mediated transphosphorylation of directly released UDP by ATP that is co-released from the same cell or released coincidently from adjacent cells (Donaldson et al., 2000; Lazarowski et al., 1997, 2000; Lazarowski and Harden, 1999). The human osteoblastic cell line SaOS-2 expresses a strong ecto-NDPK activity that acts to generate additional extracellular ATP in the presence of a γ-phosphate donor that might be UTP or

GTP (Buckley et al., 2003). Unfortunately, we were unable to detect the coexistence of UTP-consuming and UTP-generating enzymes, like the ecto-NDPK, in the kinetic studies performed in this study (Figure 11).

Membrane compartmentalization of ecto-enzymes and other components of purinergic signalling cascade have been demonstrated (reviewed by Yegutkin, 2008). According to that model, nucleotide-inactivating ecto-enzymes are located in close proximity with each other and next to nucleotides permeation sites, releasable stores and purinoceptors, presumably associated with lipid rafts and caveolae. Based on the compartmentalization model it seems reasonable to accept that released extracellular nucleotides and their derivatives are mainly concentrated on the cell surface, where they are subsequently ‘‘hand-to-hand’’ delivered for the succeeding phosphatase reactions. This important feature permits divergent cellular functions to take place at specific microdomains on the cell surface. Altogether, these multiple mechanisms for extracellular accumulation of particular P2 nucleotide agonists underscore why the characterization of potential nucleotide release mechanisms in a particular tissue or cell model should usually involve a corresponding analysis of extracellular nucleotide metabolism in that cell system (for a review, see Dubyak, 2007). In keeping with this model, our data emphasizes the value of a concerted action of UTP-consuming enzymes yielding to UDP generation and subsequent $P2Y_6$ receptors activation in determining whether osteoblast progenitors are driven into proliferation or differentiation in postmenopausal human bone. These previously unrecognized targets for local regulation of osteogenic differentiation of bone marrow-derived MSCs may prompt for novel therapeutic strategies to control human diseases where bone destruction exceeds bone formation (*e.g.*, osteoporosis, rheumatoid arthritis, osteogenesis imperfecta, fracture malunion).

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P2X7-induced zeiosis promotes osteogenic differentiation and mineralization of postmenopausal bone marrow-derived mesenchymal stem cells

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ABSTRACT

Polymorphisms of the P2X7 receptor have been associated with increased risk of fractures in postmenopausal women. Although both osteoblasts and osteoclasts express P2X7 receptors, their function in osteogenesis remains controversial. Here, we investigated the role of the P2X7 receptor on osteogenic differentiation and mineralization of bone marrow mesenchymal stem cell (MSC) cultures from postmenopausal women (age 71±3 yr, *n*=18). We focused on the mechanisms related to intracellular $[Ca^{2+}]_i$ oscillations and plasma membrane-dynamics. ATP, and the P2X7 agonist BzATP (100 μM), increased $[Ca^{2+}]_i$ in parallel to the formation of membrane pores permeable to TO-PRO-3 dye uptake. ATP and BzATP elicited reversible membrane blebs (zeiosis) in 38±1 and 70±1% of the cells, respectively. P2X7-induced zeiosis was Ca^{2+} independent, but involved phospholipase C, protein kinase C, and Rho-kinase activation. BzATP (100 μM) progressively increased the expression of Runx-2 and Osterix transcription factors by 452 and 226% (at day 21), respectively, alkaline phosphatase activity by 88% (at day 28), and mineralization by 329% (at day 43) of bone marrow MSC cultures in a Rho-kinase-dependent manner. In summary, reversible plasma membrane zeiosis involving cytoskeleton rearrangements due to activation of the P2X7-Rho-kinase axis promotes osteogenic differentiation and mineralization of bone marrow MSCs, thus providing new therapeutic targets for postmenopausal bone loss.

INTRODUCTION

Mesenchymal stem cells (MSCs) are a rare population of non-haematopoietic stromal cells present in the adult bone marrow, which exhibit extensive proliferative ability in uncommitted state while retaining great potential to differentiate into osteoblasts under appropriate conditions (Bobis et al., 2006; Pittenger et al., 1999). These unique properties make MSCs attractive candidates for bone growth and repair during aging, fracture healing, and diseases accompanied by excessive bone loss.

Extracellular adenine nucleotides acting *via* several P2 purinoceptors play important roles in the regulation of bone formation as they are constitutively released into the bone microenvironment, the levels of which significantly increase during bone injury (reviewed in Burnstock et al., 2013). Adenine nucleotides stimulate bone formation by increasing the expression of osteoblast-related genes, such as *RUNX-2* (Ciciarello et al., 2013). Released ATP also inhibits osteoclast-induced bone resorption by altering cytoskeletal structure (Miyazaki et al., 2012) and NF-kB translocation to the nucleus (Korcok et al., 2004). Communication between osteoblasts and osteoclasts involves intercellular Ca^{2+} signalling, which requires ATP release and activation of P2X7 receptors in osteoclasts (Jorgensen et al., 2002). Expression of the P2X7 receptor was also shown in a subpopulation of human differentiated osteoblasts (Gartland et al., 2001; Henriksen et al., 2006), as well as in several human osteoblast like cell lines (*e.g.*, MG-63, SaOS-2, Te85; Nakamura et al., 2000), but not in others (reviewed in Wesselius et al., 2011). These differences, together with heterogeneity on the molecular composition and activity of P2X7 receptors among human and rodent species (*e.g.*, see Roger et al., 2010), may explain the controversy in the literature regarding the predominant role of the P2X7 receptor in bone remodelling (reviewed in Burnstock et al., 2013).

Bone turnover is coordinated in adulthood. Yet later in life, especially in women after menopause, osteoclast function is increased relative to osteoblast activity, and this unbalanced cellular activity causes increased bone resorption. Epidemiological studies indicate that single nucleotide polymorphisms in the P2X7

receptor gene may favor the loss of bone mineral density and the risk of fracture in postmenopausal women (*e.g.*, see Ohlendorff et al., 2007 but also Jorgensen et al., 2012). Although the high incidence of vertebral fractures may result from increased number of osteoclasts, we hypothesized that heterogeneity of the P2X7 receptor expression among osteoprogenitors and differentiated osteoblast-like cell populations might also play a role (Burnstock et al., 2013). Therefore, this study was designed to investigate the mechanisms underlying participation of the P2X7 receptor in osteogenic differentiation and mineralization of MSCs in postmenopausal women.

The P2X7 receptor channel possesses unique characteristics among the P2X family by their much longer intracellular C-terminal tail, slow desensitization rate, and ability to switch between two open states depending on activation conditions. Basal P2X7 receptor activation leads to opening of membrane channels permeable to small cations (*e.g.*, Ca^{2+}, K^{+}, Na^{+}; Alloisio et al., 2010; Carrasquero et al., 2009; Orriss et al., 2006). During mechanical stress, tissue trauma and/or inflammation, prolonged exposure to high concentrations of ATP (>100 μM) leads to a leftward shift in agonist affinity and to opening of dilated plasma membrane pores permeable to molecules up to 900 Da (Panupinthu et al., 2007). This renders cells more prone to reversible morphological changes (*e.g.*, microvesiculation and blebbing), as observed in mouse osteoblasts (Li et al., 2005) and/or susceptible to apoptosis, as occurs in osteoclasts (North, 2002).

Given the osteogenic potential of MSCs for bone growth and repair in adulthood, and the controversy regarding the participation of the P2X7 receptor in human bone turnover, we focused our attention on the mechanisms underlying P2X7-induced membrane blebbing, pore formation, osteogenic differentiation, and mineralization of bone marrow-derived MSCs isolated from postmenopausal women as compared to younger females.

MATERIALS AND METHODS

Reagents and antibodies

ATP, ADP, adenosine 5′-[β-thio]diphosphate (ADPβS), and 2',3'-O-(benzoyl-4-benzoyl)-adenosine 5′-triphosphate (BzATP) were from Sigma-Aldrich (St. Louis, MO, USA). We obtained 3-[[5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine (A438079), chelerythrine (CHL), (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl) sulfonyl]-hexahydro-1*H*-1,4-diazepine (H1152), 2'-deoxy-N^6-methyladenosine 3',5'-bisphosphate (MRS 2179), phorbol 12-myristate 13-acetate (PMA), thapsigargin and 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) from Tocris Cookson Inc. (Bristol, UK). Primary antibodies used were anti-$P2Y_1$, anti-Runx-2 (M-70), anti-Osterix (M-15) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and anti-P2X7 (Alomone, Jerusalem, Israel). Anti-β-actin and horseradish-peroxidase-conjugated secondary antibodies were from AbCam (Cambridge, UK); Alexa Fluor 488-labelled anti-rabbit, Alexa Fluor 653-labelled anti-goat, Fluo-4NW and quinolinium 4-[3-(3-methyl-2(3*H*)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-diiodide (TO-PRO-3) were from Molecular Probes (Invitrogen, Carlsbad, USA).

Cell cultures and phenotypic characterization

Human bone marrow samples were obtained from the neck of the femur of postmenopausal women (age 71±3 yr, *n*=18) undergoing total hip arthroplasty as a result of primary osteoarthrosis. For comparison purposes, we also used bone marrow specimens from the sacrum of three younger female patients (age 14 yr) requiring bone engraftment for spinal fusion to correct scoliosis. Handling of bone marrow samples and culture of adherent cells was performed during 10–15 days (until near confluence), as described previously (Noronha-Matos et al., 2012). First subcultures were maintained for 43 days in standard culture medium [α-minimal essential medium (α-MEM) plus 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B] supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone to promote osteogenic differentiation. Phenotypic characterization of the cells (first subculture) was performed by flow cytometry (Noronha-Matos et al., 2012). These cells exhibited positive immunoreactivity against CD105 (SH2), CD29 (integrin β1) and

CD117 (tyrosine-protein kinase Kit), which have been identified as surface markers of bone marrow-derived MSCs (Bobis et al., 2006; Pittenger et al., 1999). Conversely, the cells were negative for haematopoietic surface markers, like CD14 and CD45, which have been extensively used as a good argument to distinguish bone marrow haematopoietic cells from MSCs (Baddoo et al., 2003; Pittenger et al., 1999). Thus, first passage plastic-adherent human bone marrow cells obtained under the present experimental conditions are highly enriched in multipotent MSCs.

Viability/proliferation and osteogenic differentiation of bone marrow MSCs

Cell viability/proliferation was evaluated by the 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetrasodium bromide (MTT) assay (Costa et al., 2011; Noronha-Matos et al., 2012). Data from the MTT assay correlates positively with the results measuring cell proliferation from total DNA quantification per culture well (*e.g.*, see Noronha-Matos et al., 2012). Osteogenic differentiation of bone marrow-derived MSCs was inferred from increases in alkaline phosphatase (ALP) activity and from the expression of osteogenic transcription factors, namely Runx-2 and Osterix. ALP activity was determined in cell lysates by colorimetric determination of *p*-nitrophenol (PNP) hydrolysis, as described previously; obtained values were expressed in nanomoles of PNP per minute normalized by the MTT absorbance ($nmol \cdot min^{-1} \cdot MTT^{-1}$; Noronha-Matos et al., 2012). Levels of Runx-2 and Osterix proteins were determined by Western blot analysis at culture day 7 and 21. Equal protein amounts (10 µg) loaded into SDS-PAGE (10%) gels were transferred onto a polyvinyl identifluoride membrane using a Mini-Protean Tetra Cell coupled to a Mini-Trans-Blot module (Bio-Rad, Hercules, CA, USA). Blocked membranes were incubated with anti-human primary antibodies (1:200): anti-Runx-2 (M-70, rabbit), anti-Osterix (M-15, goat). β-Actin (rabbit) was used as control. The peroxidase detection system (1.25 mM luminol; 0.2 mM coumaric acid; 0.1 M Tris, pH 8.5; and 0.032% hydrogen peroxide) was used for visualization of the immunoreactivity. Gels were analyzed using a gel blot imaging system (ChemiDoc MP; Bio-Rad). At culture day 43, calcium deposition in mineralized nodules was revealed by alizarin

red staining and photographed using a microscope (Zeiss Axiophot; Carl Zeiss, Oberkochen, Germany) equipped with a CCD camera (CoolSnap HQ; Ropers Inc., Tucson, AZ, USA) running MetaFluor 6.3 image acquisition software (Photometrics, Tuczon, AZ, USA) (23). Images were exported to ImageJ 1.37c (U.S. National Institutes of Health, Bethesda, MD, USA) for quantification of the area of bone nodules.

Single-cell intracellular calcium ($[Ca^{2+}]_i$) transients and morphological changes by confocal microscopy

$[Ca^{2+}]_i$ oscillations were evaluated in cells loaded with the fluorescent Ca^{2+} indicator, Fluo-4NW (2.5 µM), as described previously (Noronha-Matos et al., 2012). Briefly, 7-day culture dishes were mounted on the stage of a laser-scanning confocal microscope (Olympus FV1000; Olympus, Tokyo, Japan) and perfused continuously (1 ml/min) with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): 137 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.4 NaH_2PO_4, 11.9 $NaHCO_3$, and 11.2 glucose, at 37ºC (*e.g.*, see Noronha-Matos et al., 2012). In some experiments, $CaCl_2$ was omitted, and the extracellular Ca^{2+} chelator, EGTA (1 mM), was added. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by the Ca^{2+} ionophore, ionomycin (5 µM, 100% response; Noronha-Matos et al., 2012). The percentage of cells in each microscopic field (LUCPLFL 20xPH; NA 0.45) exhibiting microvesiculation and blebbing (zeiosis) before and during addition of test drugs was also determined. For cell area quantification, the morphology of the cells was compared before and after the drug exposure (6 min). The area (μm^2) of loaded cells with Fluo-4NW (2.5 µM) was measured using the FluoView Advanced software package (Olympus); images were exported to Image J 1.37c for mathematical analysis.

TO-PRO-3 dye uptake and pore formation

Membrane pore formation was monitored by measuring TO-PRO-3 dye uptake synchronously to Ca^{2+} signals in cells previously loaded with the fluorescent Ca^{2+} indicator, Fluo-4NW (2.5 µM; see above for details). After mounting culture dishes

on the stage of the confocal microscope, cells were continuously superfused with the fluorescent dye, TO-PRO-3 (1 μM), for 6 min and up to the end of the experiment. Changes in fluorescence emitted by Fluo-4NW and TOPRO-3 were detected in the time-lapse mode with the FluoView Advanced software. Fluo-4NW was excited with the 488 nm multiline Ar laser, and the emitted fluorescence was detected at 510–560 nm; TO-PRO-3 was excited with the 633 nm red He-Ne laser, and the emitted fluorescence was detected at 661 nm. Fluorescence images were collected at 20 sec intervals.

Immunofluorescence staining

Human bone marrow cells were allowed to grow in chamber slides for 7 or 21 days. Paraformaldehyde-fixed cells were incubated with the following primary antibodies: anti-$P2Y_1$ (1:50, goat) and anti-P2X7 (1:75, rabbit), in the dark for 2 h. Alexa Fluor 488 (anti-rabbit) and Alexa Fluor 653 (anti-goat) were applied as secondary antibodies for 1 h in the dark. Chamber slides were mounted with VectaShield medium and observed by confocal microscopy (Olympus FV1000; Noronha-Matos et al., 2012).

Presentation of data and statistical analysis

Results presented in this study are from bone marrow samples obtained from 18 postmenopausal female patients (age 71±3 yr); for comparison purposes, we also used bone marrow cells from 3 younger female patients (age 14 yr) in some of the experiments. For each experiment and assay, 3–8 replicas were accomplished. Data are expressed as means ± S.E.M from an *n* number of individual experiments. Data from different individuals (from the same age group) were evaluated using 1-way analysis of variance (ANOVA), and no significant differences in the pattern of cell behavior were found. Statistical differences found between control and drug-treated cultures were determined by Bonferroni's method. Values of $P<0.05$ were considered to represent significant differences.

Declaration of ethical approval

Informed consent to use the biological material, which would be otherwise discarded, was obtained. All procedures were approved by the Ethics Committees of Centro Hospitalar de Vila Nova de Gaia (University Hospital, Espinho, Portugal), Gabinete Coordenador de Investigação/Departamento de Ensino e Investigação (DEFI)–Centro Hospitalar do Porto (CHP; Porto, Portugal), and of Instituto de Ciências Biomédicas Abel Salazar (Medical School) of the University of Porto (Porto, Portugal). The investigation conforms to the principles outlined in the Declaration of Helsinki.

RESULTS

Adenine nucleotides cause $[Ca^{2+}]_i$ transients in MSCs from postmenopausal women, but only ATP and BzATP produce plasma membrane blebbing

All adenine nucleotides, ATP, BzATP, ADP and ADPβS, increased $[Ca^{2+}]_i$ above the control level (Tyrode's solution) when applied to 7 day cultures of bone marrow-derived MSCs from postmenopausal women. ATP (100 μM) caused a fast $[Ca^{2+}]_i$ rise, which typically peaked 40 sec after nucleotide addition and decayed back almost to baseline within 1–2 min; a second progressive, but of much lower amplitude, ATP-induced rise in intracellular Ca^{2+} was observed following the initial $[Ca^{2+}]_i$ transient (Figure 13A); BzATP (100 μM) elicited a sustained response that typically peaked 3 min after application and decayed back to the baseline after washout of the drug (Figure 13A). Both ATP (100 μM) and BzATP (100 μM) caused dramatic changes in cell morphology, including initial cellular retraction followed by the formation of reversible plasma microvesiculation and blebs (zeiosis; Figure 13B, C). Blebs enlarged and shrank in an asynchronous pattern with a mean lifetime of 2–3 min. When ATP and BzATP were removed, blebbing activity reversibly ceased within 1–2 min. ADP (100 μM) and its enzymatically stable analogue, ADPβS (100 μM), were also able to elicit $[Ca^{2+}]_i$ transients, but these were of much lower amplitude when compared to ATP and BzATP (Figure 13A). Nucleoside diphosphates failed to induce changes in cell membrane morphology (Figure 13B, C).

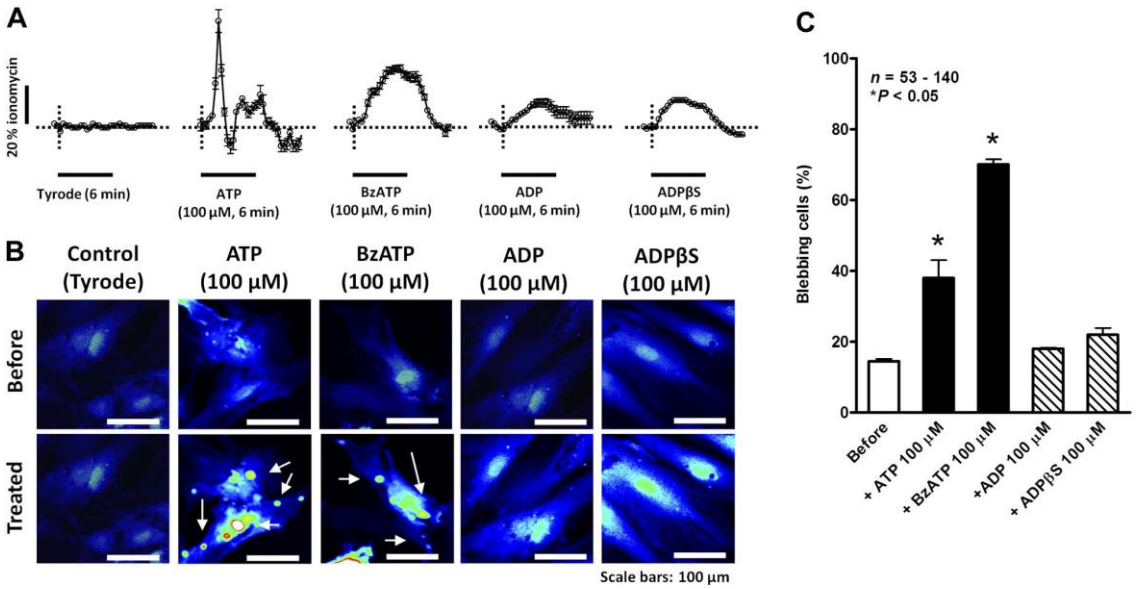

Figure 13. Adenine nucleotides cause $[Ca^{2+}]_i$ transients in MSCs from postmenopausal women, but only ATP and BzATP produce plasma membrane blebbing. Fluorescent $[Ca^{2+}]_i$ oscillations in bone marrow-derived MSCs from postmenopausal woman (age 71±3 years old) loaded with Fluo-4NW, at culture day 7, monitored in the time-lapse mode with a laser-scanning confocal microscope. Cells were challenged with Tyrode's solution (control), ATP (100 µM), BzATP (100 µM), ADP (100 µM) and ADPβS (100 µM) for 6 min (horizontal bars at the bottom of each graph). (**A**) Intracellular Ca^{2+} transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Each point represents pooled data from 3 to 5 different individuals for a total of 85 (Tyrode), 78 (ATP), 66 (BzATP), 63 (ADP) and 64 (ADPβS) cells. Error bars = S.E.M. (**B**) Representative pseudocolor micrographs of experiments shown in panel (A) obtained before and during drug treatment. Arrows indicate plasma membrane blebs formed during perfusion with ATP or BzATP. Scale bar = 100 µm. (**C**) Percentage of cells exhibiting plasma membrane blebs within each cell populations. Bars represent pooled data from *n* cells. Error bars = S.E.M. **P*<0.05 *vs*. control.

ATP and BzATP cause membrane pore formation in MSCs from postmenopausal women

Activation of ATP-sensitive P2X7 receptors may trigger the formation of large, nonspecific pores, which are permeable to molecules up to 900 Da (Panupinthu et al., 2007) *via* the recruitment of a distinct pore-forming moiety (Pelegrin and Surprenant, 2006; Verhoef et al., 2003). To test whether membrane pore formation occurs in bone marrow-derived MSC cultures from postmenopausal women challenged with ATP and BzATP, we measured the uptake of the fluorescent dye, TO-PRO-3, a carbocyanine nucleic acid stain with a molecular mass of 671 Da, simultaneously with $[Ca^{2+}]_i$ transients in cells previously loaded with Fluo-4NW. TO-PRO-3 (1 µM) dye uptake by the cells stabilized ~200 s after starting its

perfusion. No changes in $[Ca^{2+}]_i$ fluorescence were observed during TO-PRO-3 superfusion.

Figure 14 shows that the TO-PRO-3 dye uptake by postmenopausal MSCs

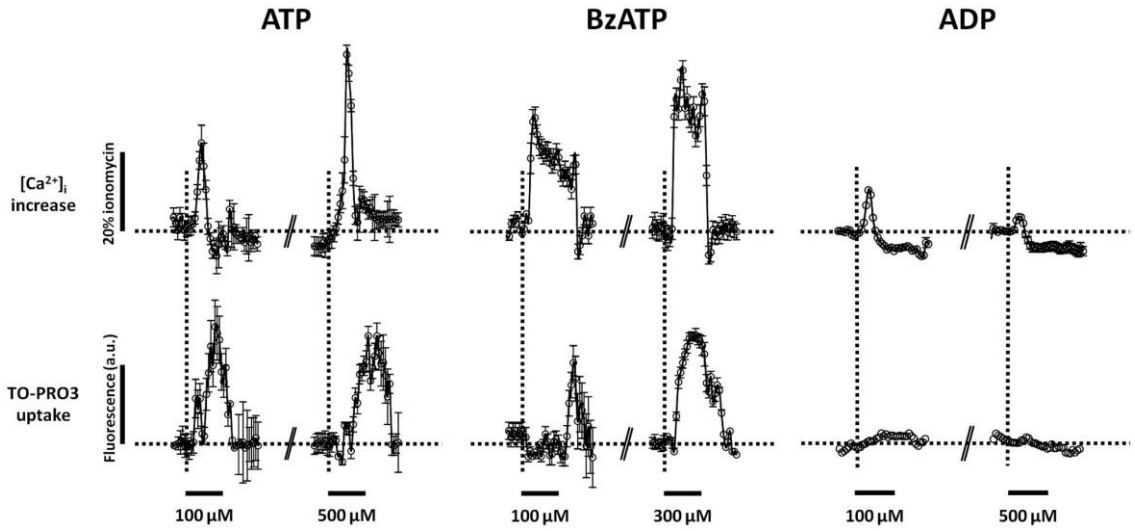

Figure 14. ATP and BzATP, but not ADP, cause membrane pore formation in MSCs from postmenopausal women. Shown are fluorescent $[Ca^{2+}]_i$ oscillations and TO-PRO-3 dye uptake in bone marrow-derived MSCs from postmenopausal woman (age 71±3 years old) at culture day 7; cells were monitored in the time-lapse mode with a laser-scanning confocal microscope. After loading with the intracellular Ca^{2+} indicator, Fluo-4NW, the cells were superfused throughout the experiment with the nucleic acid fluorescent dye, TO-PRO-3 (1 µM), in order to monitor dye uptake due to membrane pore formation. Cells were then challenged with 100 and 500 µM ATP, 100 and 300 µM BzATP and 100 and 500 µM ADP for 6 min (horizontal bars at the bottom of each graph). Data points represent pooled data from 3 individuals for a total of 73 (ATP), 99 (BzATP) and 70 (ADP) cells. Error bars = S.E.M.

occurred immediately after the initial $[Ca^{2+}]_i$ rise elicited by both ATP (100 and 500 µM) and BzATP (100 and 300 µM). TO-PRO-3 dye uptake was kept at high levels for 4–5 min beyond the initial $[Ca^{2+}]_i$ peak caused by ATP, but followed the more sustained $[Ca^{2+}]_i$ rise produced by BzATP. The magnitude of $[Ca^{2+}]_i$ transients and sustained TO-PRO-3 dye uptake was dependent on the concentration of the nucleotides. TO-PRO-3 is a membrane-impermeant DNA intercalator that binds to double-strand DNA with a much lower affinity than other carbocyanine dyes, exhibiting a dissociation constant in the micromolar range (Sovenyhazy et al., 2003). The dynamics of TO-PRO-3/DNA binding causes oscillations between 3 distinct wavelength absorption modes (514, 584, and 642 nm). This, together with a focus on the average of TO-PRO-3 dye uptake variations in whole-cell area

rather than in nuclei, may give the impression that TO-PRO-3 uptake signals are transient and reversible. Nevertheless, if one considers the nucleus as the region of interest, we could observe that the dye progressively accumulates with time after the application of ATP (100 and 500 μM) and BzATP (100 and 300 μM). Despite elevations of $[Ca^{2+}]_i$ triggered by ADP (100 and 500 μM), this nucleotide was unable to promote TO-PRO-3 dye uptake and, therefore, the formation of membrane pores in these cells.

Plasma membrane blebbing and pore formation requires P2X7 receptor activation

Despite BzATP being considered a highly effective P2X7 receptor agonist, it can bind to a number of other P2 receptors, including the human $P2Y_1$ receptor (North, 2002) that is also expressed in osteoprogenitor cells (Maier et al., 1997; Noronha-Matos et al., 2012 and Figure 15A). Preincubation of the cells with A438079 (3 μM) abolished the late component of BzATP (100 μM)-induced $[Ca^{2+}]_i$ response, while keeping a residual fast $[Ca^{2+}]_i$ rise (Figure 15B). Conversely, the selective $P2Y_1$ receptor antagonist, MRS 2179, applied in a concentration (0.3 μM) that significantly attenuated the initial $[Ca^{2+}]_i$ rise of the ATP (100 μM) response (Noronha-Matos et al., 2012), kept largely unrestrained the late component of the BzATP (100 μM) $[Ca^{2+}]_i$ response (Figure 15B). These results suggest that ATP operates a biphasic $[Ca^{2+}]_i$ response in cultured postmenopausal MSCs, which is mediated by fast desensitizing $P2Y_1$ receptors and by slow activating (nondesensitizing) P2X7 receptors, respectively (*e.g.*, see Noronha-Matos et al., 2012); the latter receptors are more likely to be activated by BzATP. Given that MRS 2179 also partially depressed the late component of BzATP-induced $[Ca^{2+}]_i$ response, one may speculate that P2X7-induced $[Ca^{2+}]_i$ rises may depend on prior activation of coexistent $P2Y_1$ receptors (Bowler et al., 1999; Buckley et al., 2001 and Figure 15A).

This scenario was different when considering BzATP-induced pore formation and plasma membrane blebbing in MSCs from postmenopausal women. The selective P2X7 receptor antagonist, A438079 (3 μM), abolished both TO-PRO-3

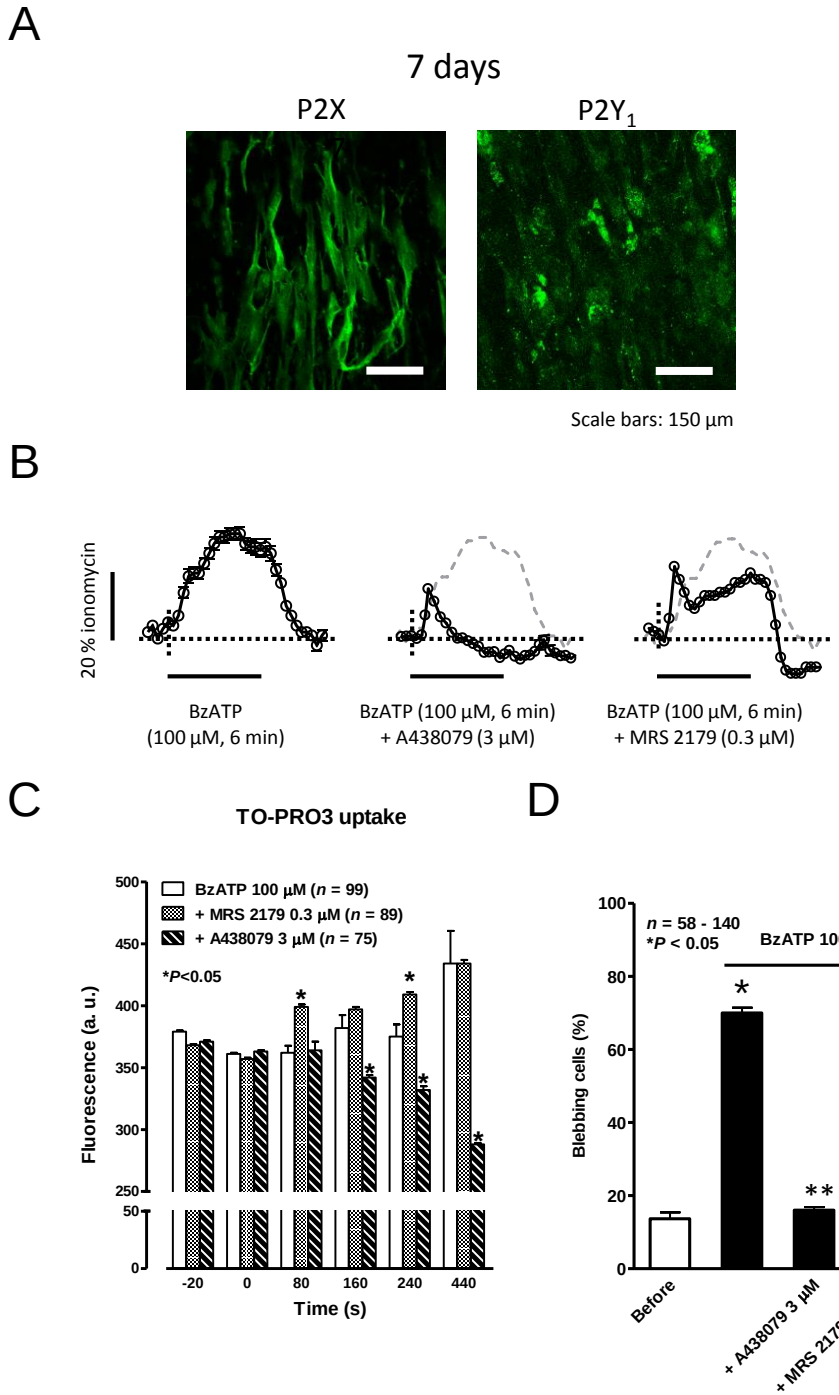

Figure 15. Blebbing and pore formation in MSCs from postmenopausal women requires P2X7 receptor activation. **(A)** Immunocytochemical detection of P2X7 and $P2Y_1$ receptors on 7 day cultures of MSCs. Confocal micrograph images representative of 3 individual experiments. Scale bars 150 µm. *B–D*) Fluorescent $[Ca^{2+}]_i$ oscillations **(B)**, TO-PRO-3 dye uptake **(C)**, and plasma membrane blebbing **(D)** were monitored simultaneously by confocal microscopy in the time-lapse mode. After loading the cells with Fluo-4 NW, the superfusion fluid was supplemented with TO-PRO-3 (1 µM).

dye uptake (Figure 15C) and plasma membrane blebbing (Figure 15D) caused by

Figure 15. *(Continued)* Cells were challenged with BzATP (100 μM, for 6 min) in the absence and in the presence of A438079 (3 μM, a selective P2X7 receptor antagonist) or MRS 2179 (0.3 μM, a selective $P2Y_1$ receptor antagonist). (B) Each point represents pooled data from 3–5 individuals for a total of 32 cells (BzATP), 80 cells (BzATP plus A438079), and 50 cells (BzATP plus MRS 2179). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). (C) Bars represent pooled data from 3 individuals for *n* cells. Values are in arbitrary fluorescence units. **P*<0.05 *vs*. BzATP alone. (D) Percentage of cells exhibiting plasma membrane blebs within each cell populations. Bars represent pooled data from *n* cells. Error bars = S.E.M. **P*<0.05 *vs*. control (before addition of drugs); ***P*<0.05 *vs*. BzATP alone.

BzATP (100 μM), whereas MRS 2179 (0.3 μM) was ineffective. These results suggest that despite the role the $P2Y_1$ receptor might play in $[Ca^{2+}]_i$ mobilization caused by BzATP, this receptor lacks effect on membrane cell dynamics, which may justify the lack of action of the $P2Y_1$ receptor ligands, ADP (100 μM) and ADPβS (100 μM), on membrane zeiosis (see Figure 13B, C).

P2X7-induced plasma membrane blebbing is independent of Ca^{2+}, but involves activation of phospholipase C (PLC), protein kinase C (PKC), and Rho-associated kinase in MSCs from postmenopausal women

Although the pharmacology and channel properties of the P2X7 receptor have been studied extensively, signal transduction pathways are relatively unknown. It has been demonstrated that P2X7 receptors signal through phospholipase D (PLD) and phospholipase A_2 (PLA_2) in osteoblasts from newborn rats (Panupinthu et al., 2007). These researchers showed that lysophosphatidic acid (LPA) resulting from PLD activity acts on specific receptors on osteoblasts to cause membrane cell blebbing *via* a pathway dependent on Rho-associated kinase (Panupinthu et al., 2007). The interplay between PKC and Rho-kinase activation has been hypothesized, although this interaction has not been experimentally proven. Here, we examined whether inhibition of Rho-associated kinase using H1152 (3 μM; Sasaki et al., 2002; Tamura et al., 2005) or inhibition of PKC by CHL (5 μM; Herbert et al., 1990) affected membrane cell blebbing in cultured postmenopausal MSCs challenged with BzATP (100 μM). Figure 16Ai shows that BzATP (100 μM) -induced plasma membrane blebbing was prevented by both H1152 (3 μM, Figure 16Aii) and CHL (5 μM, Figure 16Aiii); statistical analysis of these experiments is shown in Figure 16B. Despite their effect on membrane cell blebbing, the two

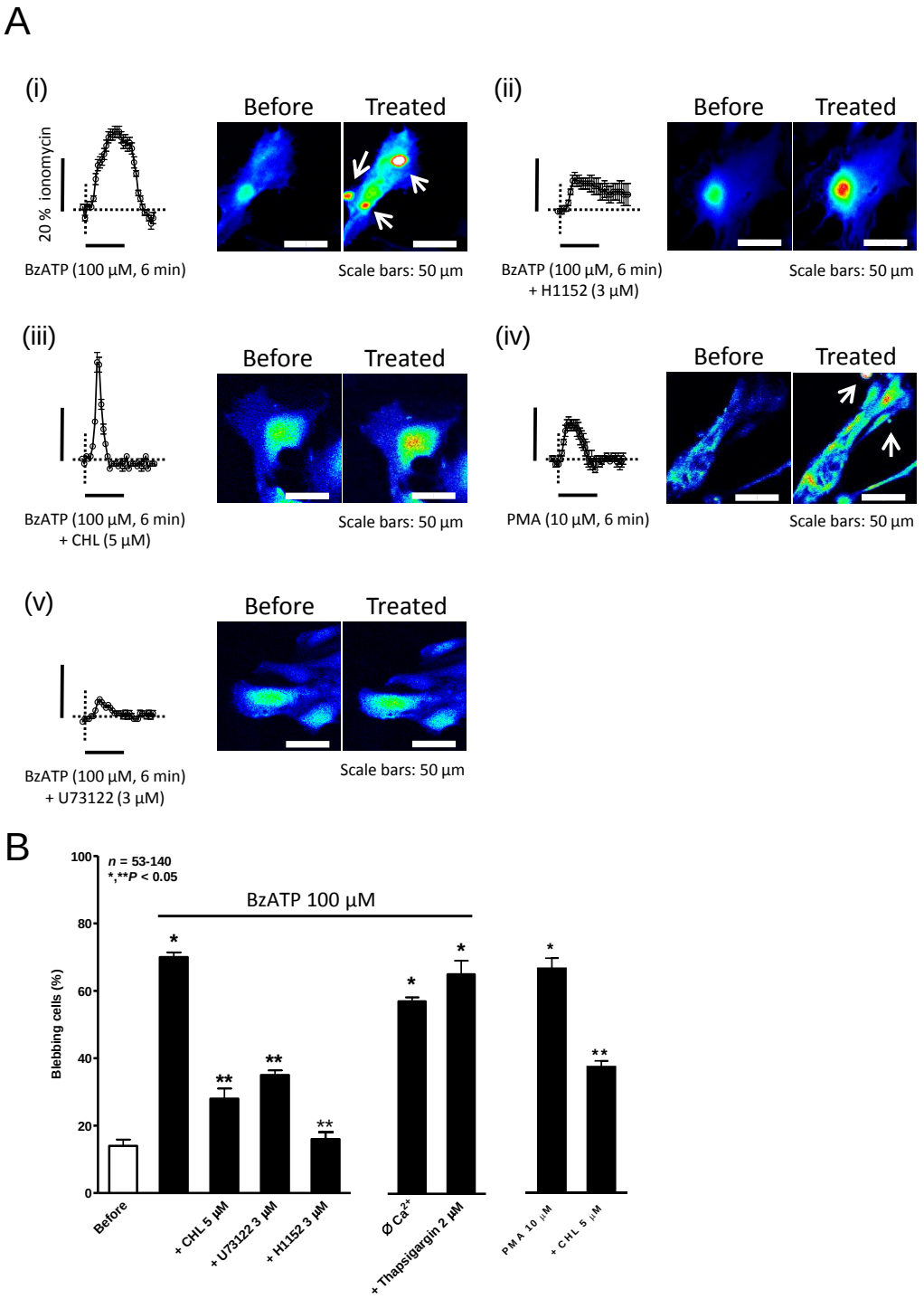

Figure 16. P2X7-induced membrane cell dynamics require activation of PLC, PKC and Rho-associated kinase in MSCs from postmenopausal women. $[Ca^{2+}]_i$ oscillations and membrane blebs were monitored by confocal microscopy in the time-lapse mode in MSCs loaded with Fluo-4 NW, at culture day 7. (**A**) Left panels: $[Ca^{2+}]_i$ transients calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response).

enzyme inhibitors differently affected BzATP (100 µM)-induced $[Ca^{2+}]_i$ response.

Figure 16. *(Continued)* $[Ca^{2+}]_i$ oscillations and membrane blebs were monitored by confocal microscopy in the time-lapse mode in MSCs loaded with Fluo-4NW, at culture day 7. (**A**) Left panels: $[Ca^{2+}]_i$ transients calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). Each point represents pooled data from 3-5 different individuals for a total of 30 cells (BzATP 100 μM, Ai), 61 cells (BzATP 100 μM plus H1152 3 μM, Aii), 80 cells (BzATP 100 μM plus CHL 5 μM, Aiii), 36 cells (PMA 10 μM, Aiv) and 65 cells (BzATP 100 μM plus U73122 3 μM, Av). Error bars = S.E.M. Right panels: pseudocolor micrographs obtained before and during drug treatment. Arrows indicate membrane blebs formed during perfusion with BzATP (100 μM) and PMA (10 μM). Scale bars = 50 μm. (**B**) Percentage of cells exhibiting membrane blebs within each situation, along with the effect of BzATP (100 μM) in a Ca^{2+}-free solution (CaØ + 1mM EGTA) obtained in the absence or in the presence of thapsigargin (2 μM), used to deplete intracellular Ca^{2+} reservoirs. Bars represente pooled data from *n* cells (3-5 individuals). Error bars = S.E.M. **P*<0.05 vs. control (before addition of drugs); ***P*<0.05 vs. BzATP or PMA alone.

Inhibition of Rho-associated kinase by H1152 (3 μM) globally depressed $[Ca^{2+}]_i$ transients caused by BzATP (100 μM; Figure 16Aii). Conversely, CHL (5 μM) prevented the sustained component of BzATP (100 μM)-induced $[Ca^{2+}]_i$ response, while keeping almost unaltered the initial $[Ca^{2+}]_i$ rise (Figure 16Aiii). To confirm the PKC involvement in zeiosis, we tested the effect of a phorbol ester activator of PKC, PMA (5 μM; Castagna et al., 1982). PMA mimicked the effect of BzATP on the total number of cells exhibiting membrane blebbing, while producing a smaller $[Ca^{2+}]_i$ response than the nucleotide (Figure 16Aiv, B). Data suggest that PKC and Rho-associated kinase effectively mediate membrane cell blebbing downstream P2X7 receptor activation.

As mentioned above, P2X7 activation may lead to the production of LPA and subsequent activation of the Gq-coupled LPA1 receptor, which may mediate blebbing in osteoblasts and, thus, osteogenic differentiation during skeletal development in rats (Panupinthu et al., 2007). Stimulation of Gq-coupled receptors leads, most commonly, to PLC activation and increases in plasma membrane diacylglycerol (DAG) and cytosolic IP3 levels (Bleasdale et al., 1990). DAG would otherwise activate PKC. Pretreatment of the cells with U73122 (3 μM) significantly reduced BzATP-induced $[Ca^{2+}]_i$ response, as well as membrane cell blebbing; under these conditions, only 35±1% of the cells exposed to the nucleotide showed blebbing (Figure 16Av, B). Overall, these results suggest that P2X7-induced plasma membrane blebbing involves activation of PLC, PKC, and Rho-associated kinase in MSCs from postmenopausal women.

Taking into consideration that BzATP (100 μM) elicits plasma membrane blebbing irrespective of the pattern obtained for the $[Ca^{2+}]_i$ response, we decided to perform similar experiments in the absence of external Ca^{2+} (plus EGTA, 1 mM) and after depleting the intracellular Ca^{2+} stores with thapsigargin (2 μM). BzATP (100 μM)-induced membrane cell blebbing was still observed in 57±1% of the cells tested in Ca^{2+}-free Tyrode's solution supplemented with the Ca^{2+} chelator, EGTA (1 mM; Figure 16B). BzATP (100 μM) accelerated Ca^{2+} influx from the extracellular milieu on readmission of $CaCl_2$ to control levels (1.8 mM) in the Tyrode's solution. Under control conditions, $[Ca^{2+}]_i$ peaked to 74±3% (*n*=48 cells) of the ionomycin response in 200 sec, while the maximal $[Ca^{2+}]_i$ load (76±2% of the ionomycin response, *n*=44 cells) was anticipated by 80 sec ($P<0.05$) when the cells were preincubated with the P2X7 receptor agonist. The specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (2 μM; Thastrup et al., 1990), failed to affect the formation of membrane cell blebs caused by BzATP (100 μM), which were observed in 65±1% of tested cells (Figure 16B). This reinforces the idea that P2X7-induced membrane cell blebbing is a Ca^{2+}-independent phenomenon in MSCs from postmenopausal women.

P2X7-induced changes in the morphology of MSCs from postmenopausal women depend on PLC, PKC, and Rho-associated kinase

The mechanism underlying membrane cell blebbing is thought to involve actomyosin contraction, but the initiating events are poorly understood. Interestingly, cell shape and cytoskeletal tension generated by actomyosin have been shown to regulate osteogenic differentiation of human MSCs (McBeath et al., 2004). In the same set of previous experiments, we were able to measure the changes in cell area on application of BzATP (100 μM) in the absence and in the presence of the selective P2X7 antagonist A438079 (3 μM), the PLC inhibitor U73122 (3 μM), the PKC inhibitor CHL (5 μM), and the Rho-associated kinase inhibitor H1152 (3 μM). Figure 17A shows a representative image of a cell loaded with the Ca^{2+} indicator, Fluo-4NW, and the corresponding differential interference contrast (DIC) image, just before and during BzATP (100 μM) application. One can

appreciate a clear retraction of the cell synchronous to application of the nucleotide along with the formation of plasma membrane blebs (Figure 17A, magnified inset). On average, the total area of the cells shrank by 316±93 μm^2 (*n*=67) after exposure to BzATP (100 µM) for 6 min. Contraction of the cells caused by BzATP (100 µM) was significantly attenuated in the presence of A438079 (3 µM), H1152 (3 µM), chelerythrine (5 µM), and U73122 (3 µM) (Figure 17B). These results suggest that P2X7-induced changes in the morphology of MSCs from postmenopausal women depend on PLC, PKC, and Rho-associated kinase. The involvement of PKC in cell shrinkage was confirmed using PMA (10 µM). Like that observed for membrane cell blebbing, PMA (10 µM) also mimicked the effect of BzATP (100 µM) on cell retraction. A reduction of the total cell area by 425±133 μm^2 (*n*=32) was observed in the presence of PMA (10 µM), an effect that was completely reverted by CHL (5 µM, *n*=41), strengthening the involvement of PKC in cell shape changes (Figure 17B). P2X7-induced morphological changes in MSCs from postmenopausal women are mostly Ca^{2+}-independent, because the cell shrinkage effect of BzATP (100 µM) was enhanced, rather than reduced, to 511±56 μm^2 (*n*=97) in the absence of extracellular Ca^{2+} (plus 1 mM EGTA) and depletion of internal Ca^{2+} stores with thapsigargin (2 µM) had no significant effect (316±74 μm^2, *n*=79).

Osteogenic commitment of MSCs from postmenopausal women is impaired as compared to younger females

Cell viability/proliferation (given by the MTT assay) of postmenopausal MSCs grown in osteogenesis-inducing conditions gradually increase until day 28, a situation that was not different from that observed with the cells isolated from younger females (*e.g.*, see Noronha-Matos et al., 2012). Significant (*P*<0.05) differences were, however, observed regarding the osteogenic differentiation profile between the two groups (Figure 18). Cell cultures from the younger group (age 14 yr, *n*=3) exhibited higher ALP activity normalized by the number of viable cells (MTT value) than the postmenopausal group (age 71±3 yr, *n*=18). ALP activity in the younger group of patients reached a maximum at day 14 (Figure

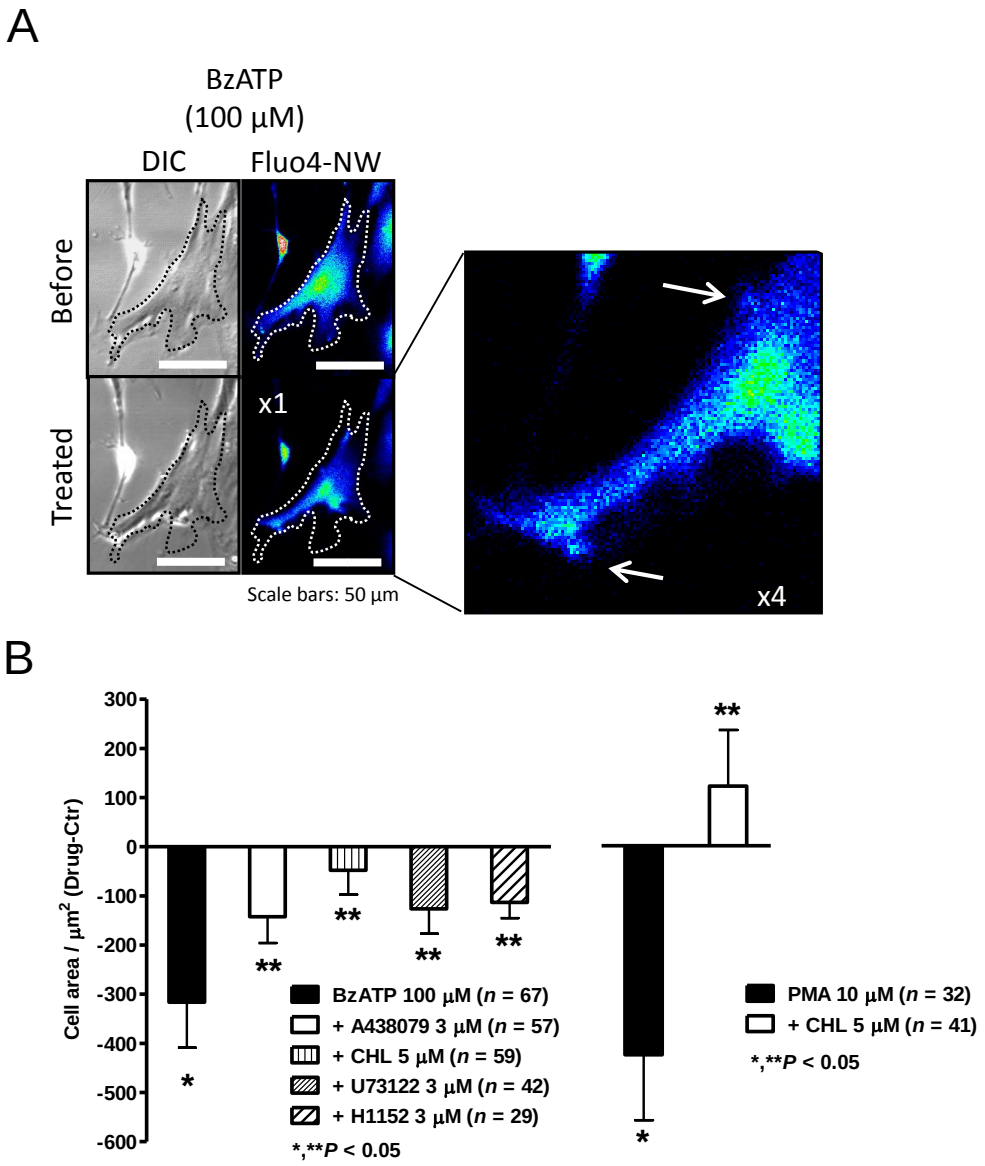

Figure 17. PLC, PKC and Rho-associated kinase are involved in cell retraction induced by activation of the P2X7 receptor in MSCs from postmenopausal women. (**A**) Representative confocal micrographs of postmenopausal MSCs loaded with Fluo-4NW, obtained at culture day 7 just before and during perfusion with BzATP (100 µM). Arrows indicate high magnification plasma membrane blebs. Scale bars = 50 µm. Pseudocolor images represent $[Ca^{2+}]_i$ levels; cell boundaries are best appreciated by differential interference contrast (DIC) microscopy. (**B**) Variation of cell area (μm^2) after application of drugs as compared to the control situation (negative values indicate cell retraction). Bars represents pooled data from 3-5 individuals for a total of 67 cells (BzATP 100 µM), 57 cells (BzATP 100 µM plus A438079 3 µM), 59 cells (BzATP 100 µM plus CHL 5 µM), 42 cells (BzATP 100 µM plus U73122 3 µM), 29 cells (BzATP 100 µM plus H1152 3 µM), 32 cells (PMA 10 µM), and 41 cells (PMA 10 µM plus CHL 5 µM). Error bars = S.E.M. **P*<0.05 vs. control (before addition of drugs); ***P*<0.05 vs. BzATP or PMA alone.

18A), whereas in the postmenopausal group, ALP activity increased slowly, yet progressively, with incubation time until day 28.

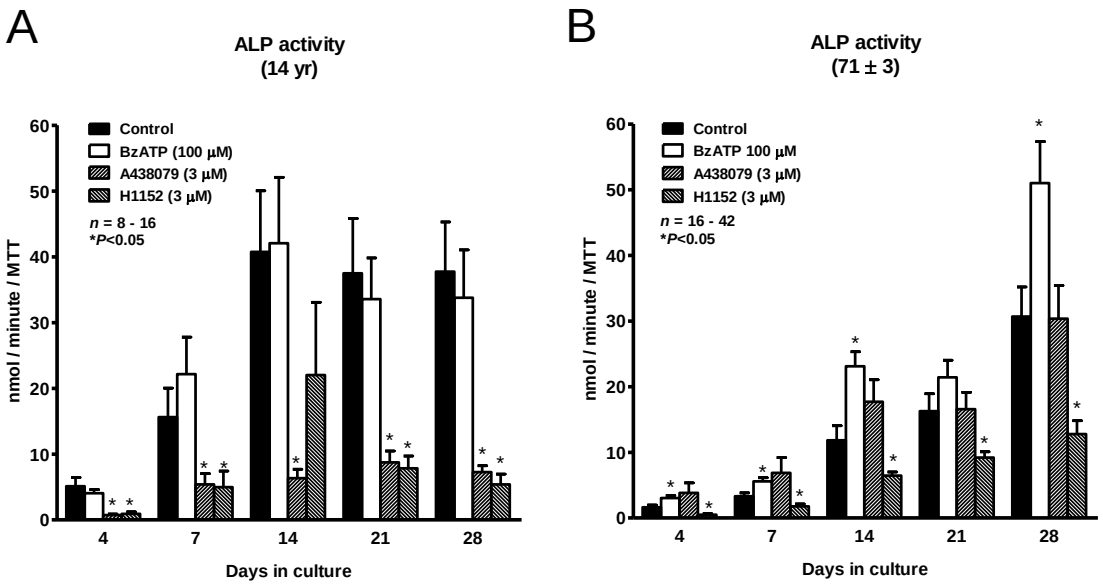

Figure 18. Activity of ALP in cell lysates of bone marrow-derived MSCs from young females (**A**) and postmenopausal women (**B**) exposed to BzATP (100 µM), A438079 (3 µM) and H1152 (3 µM) for 28 days. Ordinates are nanomoles of *p*-nitrophenol (*p*NP; at 405 nm) produced per minute from *p*-nitrophenylphosphate (*p*NPP) catalysed by ALP normalized by MTT values at given time points. Bone marrow samples were obtained from 3 young female patients (age 14 yr) and 5 postmenopausal women (age 71±3 yr); 3-8 replicas were performed in each individual experiment. Error bars = S.E.M. **P*<0.05 *vs*. control.

Activation of the P2X7 receptor on postmenopausal MSCs resumes the osteogenic differentiation profile detected in younger females

The effects of the P2X7 receptor activation on osteoblast cell cultures have been addressed in several studies, often with conflicting results (for a review, see Burnstock et al., 2013). For instance, the P2X7 receptor has been implicated in osteogenesis promotion in newborn rats (Panupinthu et al., 2007) and mediates shockwave-induced osteogenic differentiation of MSCs from healthy human volunteers (Sun et al., 2013). Conversely, other researchers observed reductions in ALP activity and bone mineralization in osteoblast cultures isolated from 2-days-old neonatal rats (Orriss et al., 2012). In SaOS-2 cells, a human osteosarcoma cell line, BzATP favoured osteoblast apoptosis (Gartland et al., 2001). This dispute

prompted us to investigate the role of the P2X7 receptor on osteogenic differentiation of MSCs from postmenopausal women, given that the gain of function of this receptor has been associated with increased bone mass (Husted et al., 2013).

Treatment of MSCs with BzATP (100 μM) significantly increased the ALP activity above the control level. This was verified in MSC cultures from postmenopausal women (Figure 18B), but not in those from younger females (Figure 18A). On the contrary, the P2X7 receptor antagonist, A438079, failed to modify the ALP activity in MSC cultures from postmenopausal women (Figure 18B), when it was applied in a concentration (3 μM) that almost prevented osteogenic differentiation in cells from younger female patients (Figure 18A). Inhibition of Rho-kinase with H1152 (3 μM) significantly decreased the ALP activity measured in MSC cultures from both age groups (Figure 18A, B). The enhancement of ALP activity caused by BzATP (100 μM) in postmenopausal MSCs was significantly attenuated by blocking the P2X7 receptor with A438079 (3 μM). Coapplication of BzATP (100 μM) with the Rho-associated kinase inhibitor, H1152 (3 μM), abolished the increase in ALP activity observed with the nucleotide alone in cells from both age groups (Figure 18A, B). These findings suggest that P2X7 receptor activation promotes osteogenic differentiation in MSCs from postmenopausal women through a mechanism that involves stimulation of Rho associated kinase. Data also indicate that P2X7 receptors are tonically activated by endogenous ATP in cells from young females, but the osteogenic differentiating tone of the P2X7 receptor seems to be impaired in MSC cultures from postmenopausal women, probably because the nucleotide does not reach high enough concentrations in the cell microenvironment to activate this receptor.

Runx-2 and Osterix are important transcription factors involved in bone formation. Runx-2 is involved in osteoblastic differentiation and skeletal morphogenesis; it is essential for the maturation of osteoblasts and ossification, both intramembranous and endochondral. It binds to a number of enhancers and promoters, including osteocalcin, osteopontin, bone sialoprotein and type I collagen (Ducy et al., 1997; Kern et al., 2001). Osterix acts downstream of Runx-2

and is a zinc-finger-containing transcription factor, which is essential for embryonic osteoblast differentiation and bone formation (Nakashima et al., 2002). In this study, we collected total cell lysates for Runx-2 and osterix protein quantification by Western blot analysis. Western blot gels (Figure 19A, B) show that postmenopausal MSCs at culture day 7 and 21 express the two osteogenic differentiation markers, Runx-2 and Osterix. BzATP (100 µM) increased the expression of Runx-2 and osterix proteins from day 7 to 21 by ~452 and 226%, respectively (Figure 19C). In addition to the increments in ALP activity observed with BzATP, the ATP analogue increased the expression of important bone transcription factors, Runx-2 and Osterix, indicating that P2X7 receptor promotes osteogenic commitment of MSCs from postmenopausal women.

P2X7 receptor activation promotes mineralization of postmenopausal MSC cultures

The ultimate goal of bone-forming osteoprogenitors is the ability to mineralize the extracellular matrix *via* ALP activity. In this context, we performed histochemical assays using the Alizarin red staining at culture day 43 in order to identify extracellular calcium deposits corresponding to areas of mineralization of the cultures. We compared data obtained in the absence and in the presence of BzATP (100 µM) in MSCs from postmenopausal women and younger females. In control conditions, the total mineralized area (Figure 20A) and the total number of bone nodules formed per culture well (Figure 20B) were significantly higher in the young female group as compared to MSC cultures from postmenopausal women. Continuous application of BzATP (100 µM) significantly increased the total mineralized area and the total number of bone nodules formed per culture well in both age groups (Figure 20). The mineralization effect of the P2X7 receptor agonist was more notorious in the young female group, but BzATP (100 µM) was still capable of increasing by ~4-fold mineralization of MSCs cultures from postmenopausal women as compared to the control level. It is also worth noting that both total mineralized area and bone nodule formation in postmenopausal MSC cultures treated with BzATP (100 µM) overcame mineralization parameters

determined in control conditions (no drugs added) for the young female group

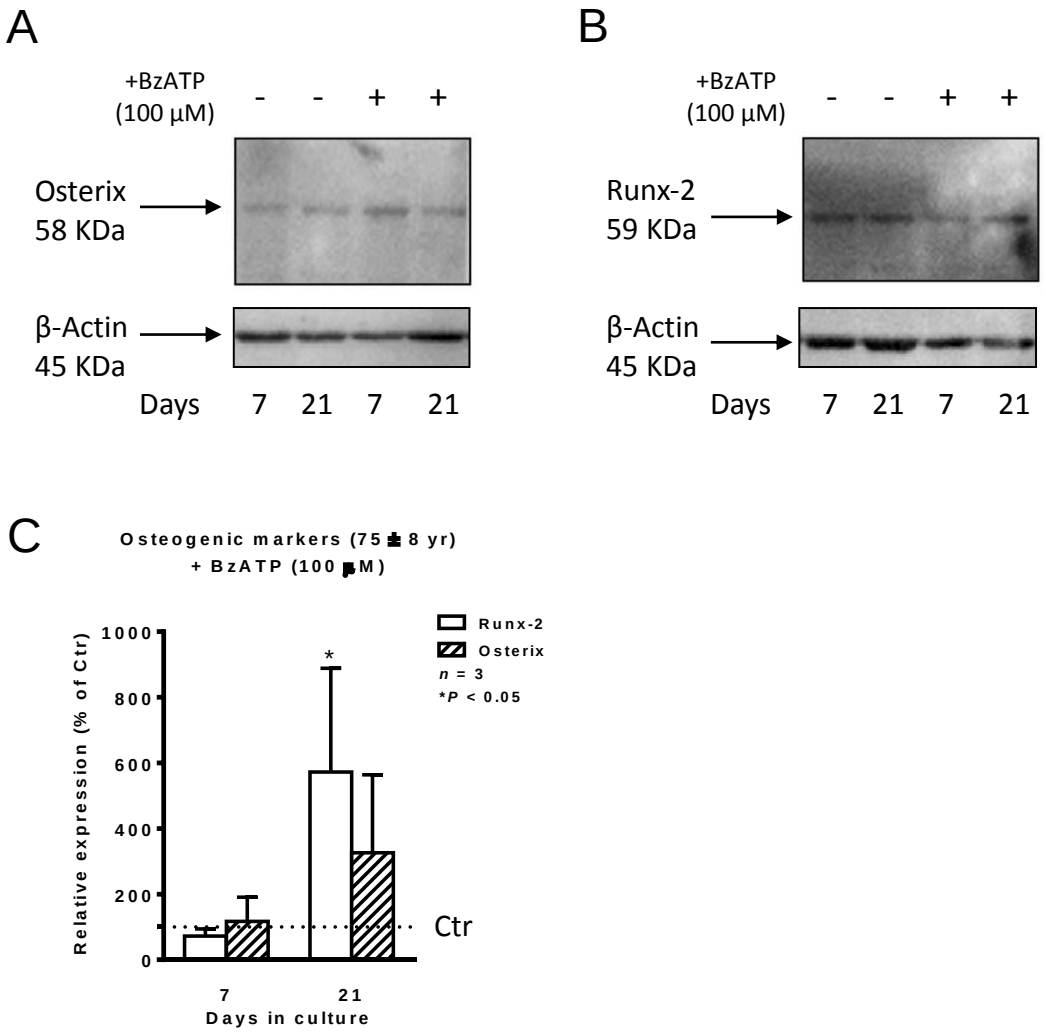

Figure 19. (A), (B) Western blot analysis of Osterix (58 KDa; A) and Runx-2 (59 KDa; B) transcription factors in MSCs from 3 postmenopausal women (age 75±8 yr) at culture day 7 and 21: influence of BzATP (100 µM). β-Actin (45 KDa) was used as control. (**C**) Relative expression of Osterix and Runx-2 in postmenopausal MSCs as a percentage of control values detected without adding BzATP (dashed horizontal line). Error bars = S.E.M. **P*<0.05 *vs*. control values obtained in the absence of BzATP.

(Figure 20). BzATP (100 µM)-induced mineralization of MSCs cultures was fully prevented in the presence of the selective P2X7 antagonist, A438079 (3 µM). Likewise, inhibition of the Rho-associated kinase with H1152 (3 µM) also abolished mineralization of the cultures caused by BzATP (100 µM). Interestingly, mineralization of the cultures was slightly reduced when A438079 (3 µM) and H1152 (3 µM) were applied alone (Figure 20); differences reached statistical

significance in the young female group, thus coinciding with the findings obtained

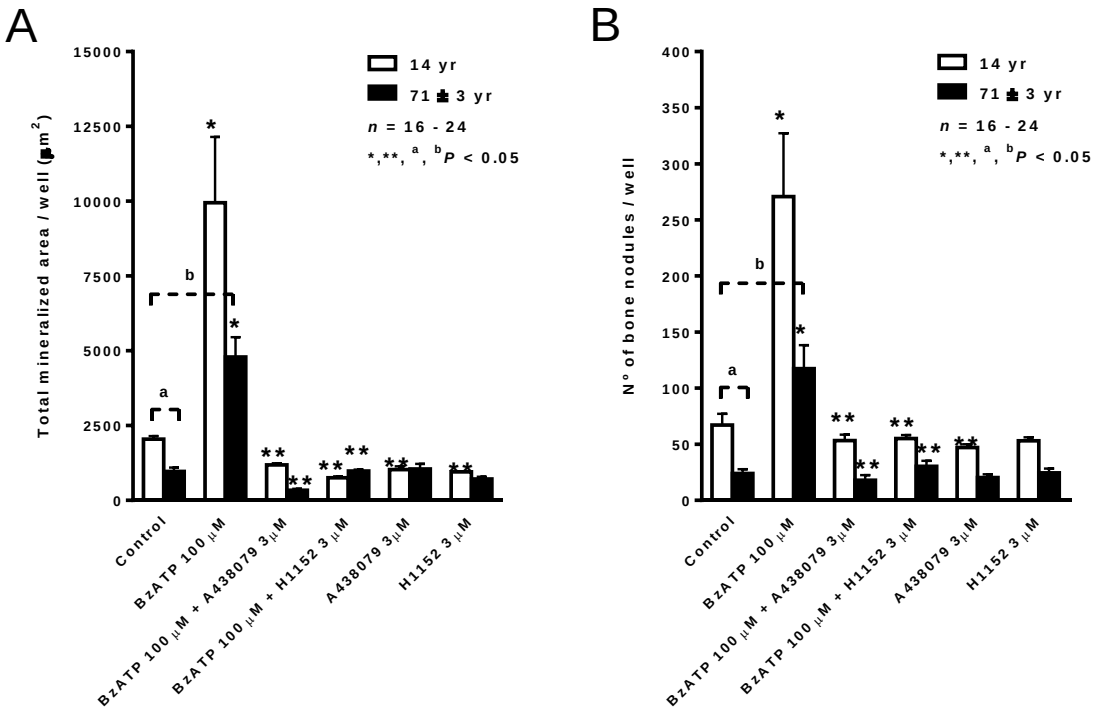

Figure 20. P2X7 receptor activation promotes mineralization of bone marrow-derived MSCs cultures from young females (age 14 yrold, *n*=3) and postmenopausal women (age 71±3 yr, *n*=18). Cultures were continuously exposed to BzATP (100 μM) in the absence and in the presence of A438079 (3 μM) or H1152 (3 μM), for 43 days; effects of H1152 (3 μM) and A438079 (3 μM) applied alone are also shown. Calcium deposition was assessed by the Alizarin red test. Results are expressed as total mineralized area (μm^2) **(A)** and total number of bone nodules per culture well (**B**). Bars represent pooled data from *n* individual experiments. Error bars = S.E.M. **P*<0.05 *vs*. control (no drugs added); ***P*<0.05 *vs*. BzATP alone; ^{a}*P*<0.05; ^{b}*P*<0.05

for the ALP activity (see Figure 18). Data suggest that tonic activation of the P2X7 receptor by endogenously released ATP favours mineralization of MSCs, *via* a mechanism involving downstream activation of Rho-associated kinase.

DISCUSSION

We present here data showing that activation of the P2X7 receptor in postmenopausal MSCs causes plasma membrane blebbing and the formation of large pores permeable to high-molecular-mass (up to 900 Da) compounds in a Ca^{2+}-independent manner. Most notably, P2X7 receptors activation favoured

osteogenic commitment of postmenopausal MSCs measured as increases in alkaline phosphatase activity and in the expression of Runx-2 and Osterix transcription factors. Mineralization of postmenopausal MSC cultures in the presence of the P2X7 agonist, BzATP, resumed the pattern observed in MSC cultures from younger (14-yr-old) females. Results also support the involvement of PLC, PKC, and Rho-associated kinase downstream the P2X7 receptor activation as important mediators of osteogenic differentiation and mineralization of MSCs from both young females and postmenopausal women. Our findings emphasize the putative clinical implications of the P2X7 receptor and its downstream PLC/PKC/Rho kinase pathway in bone loss disorders.

Previous reports implicate ATP, *via* the P2X7 receptor, in membrane zeiosis observed in hepatocyte (Nicotera et al., 1986), thymocyte (Zheng et al., 1991), and macrophage (Pelegrin and Surprenant, 2006; Verhoef et al., 2003) cell lines. This was more recently detected in osteoblasts from newborn rats (Panupinthu et al., 2007). Here, we add compelling evidence showing that ATP and the preferential P2X7 receptor agonist, BzATP, favour the uptake of the high-molecular mass dye TO-PRO-3 (671 Da) in parallel to the formation of plasma membrane blebs and cell shrinkage in postmenopausal MSCs. Involvement of the nondesensitizing P2X7 receptor (Pelegrin and Surprenant, 2006; Verhoef et al., 2003) was confirmed by selectively blocking the effects of BzATP with A438079. In contrast to changes in $[Ca^{2+}]_i$ transients (Soulet et al., 2005), blockade of the $P2Y_1$ receptor with MRS 2179 was devoid of effect on BzATP-induced membrane cell blebbing and TOPRO-3 dye uptake, thus rejecting the involvement of ADP-sensitive receptors (namely the $P2Y_1$ receptor) on postmenopausal MSCs membrane plasticity.

Using osteoblasts from newborn rats, Panupinthu *et al*. (Panupinthu et al., 2007) showed that blebbing was fully reversible on removal of BzATP and that cells respread with extension of pseudopods and peripheral ruffling. These observations imply a clearcut distinction between P2X7-induced blebbing in osteoprogenitors and apoptotic blebbing seen in osteoclasts. Moreover, reversible membrane plasticity most likely involves cytoskeletal tension generated by

actomyosin filaments, which has been implicated in osteogenic commitment of human MSCs (McBeath et al., 2004). Here, we proved that reversible P2X7-induced membrane plasticity phenomena are also relevant in postmenopausal MSCs undergoing osteogenic differentiation.

Our data support the hypothesis that P2X7-induced membrane blebbing and cell shrinkage involve downstream activation of PLC, PKC, and Rho-associated kinase in MSCs from postmenopausal women. Notably, BzATP-induced $[Ca^{2+}]_i$ transients were also modified by inhibitors of PLC, PKC, and Rho-kinase, respectively U73122, CHL, and H1152. Inhibition of PKC with CHL affected predominantly the late component of BzATP induced $[Ca^{2+}]_i$ response, which coincides with the timing expected for the occurrence of large-conductance pore formation due to prolonged exposure to P2X7 receptor agonists, as suggested previously (Faria et al., 2010). Data from this study are in agreement with previous findings demonstrating that Rho-kinase inhibition prevents membrane cell blebbing (Morelli et al., 2003; Panupinthu et al., 2007). Dependency on Rho-associated kinase activity has been detected in other cell types (Davies et al., 2000; Morelli et al., 2003; Verhoef et al., 2003), showing that this pathway has a broad influence spectrum on P2X7-induced zeiosis besides osteogenic differentiation. It has been demonstrated that PKC and Rho-kinase coexist on the same intracellular signalling pathway in porcine coronary artery (Kandabashi et al., 2003). Rho-A was also shown to be involved in PTH-stimulated PKC translocation and in IL-6 promoter expression in the rat osteosarcoma cell line, UMR-106 (Radeff et al., 2004). Focal adhesion and stress fibre formation stimulated by Thy-1 was PKC-α and Rho-A dependent in a rat astrocytic cell line (Avalos et al., 2009). Others showed the involvement of both P2X7 and PKC on large-conductance channel formation in 2BH4 cells and peritoneal macrophages using a patch-clamp technique (Faria et al., 2010). Regarding our belief that PLC functions downstream of P2X7 receptor activation, as it could be blocked by U73122, other researchers suggested the involvement of Gq-coupled receptor LPA1 in membrane cell blebbing that results from increases in DAG levels and PKC activation (Panupinthu et al., 2007).

Interestingly, we found that P2X7-induced morphological changes were largely independent of Ca^{2+} in postmenopausal MSCs, given that removal of extracellular Ca^{2+} (plus EGTA) and prevention of intracellular Ca^{2+} recruitment with thapsigargin failed to prevent blebbing. Previous reports detected a similar percentage of cells exhibiting membrane blebbing in the presence of physiological concentrations of Ca^{2+} and in a Ca^{2+}-free buffer, but higher concentrations of BzATP were required in the former case, since low concentration of divalent cations favour the P2X7 receptor activation (Panupinthu et al., 2007). The lack of Ca^{2+} involvement on morphological changes triggered by the P2X7 receptor in human MSCs differs from that observed in rats (Panupinthu et al., 2007), most probably because the human receptor lacks the calmodulin binding domain at the C terminus (Roger et al., 2010). Thus, most probably, membrane plasticity phenomena resulting from activation of the P2X7 receptor rely on the release of LPA from cell membranes through the action of PLD and PLA_2 (Panupinthu et al., 2007 and 2008).

Rho-associated kinase has been shown to cause phosphorylation of myosin regulatory light chain, which in turn controls actomyosin filament assembly and contraction (Coleman et al., 2001; Leverrier and Ridley, 2001). Here, we showed that postmenopausal MSCs undergoing osteogenic differentiation depend on morphological changes triggered by the P2X7–Rho-associated kinase axis. PKC and PLC are equally involved, since inhibition of these enzymes attenuates cell shrinkage. Other researchers have shown that rat osteoclast cell retraction induced by calcitonin is PKC dependent (Cheewatrakoolpong et al., 2005). In fact, PMA was able to induce postmenopausal MSC retraction, reinforcing the PKC involvement in this phenomenon. Contraction of cortical actomyosin filaments causes their focal detachment from the plasma membrane and increases cytoplasmic pressure, both of which lead to the formation of blebs. Since blebbing reflects vigorous actomyosin contraction, it is plausible that there may be a role for this phenomenon in cell migration or assembly of extracellular matrix by MSCs underlying osteogenic commitment in the bone microenvironment. Interestingly, most prevalent variants of P2X7 receptor resulting from alternative splicing have large deletions in the long C-terminal tail, which is critical for receptor

oligomerization, translocation to the plasma membrane, and functional coupling to intracellular signalling cascades (*e.g.*, phospholipases, RhoA/ROCK, and epithelial membrane protein 2) and actin cytoskeleton involved in cell blebbing and pore formation (Cheewatrakoolpong et al., 2005). Variation of the P2X7 receptor structure may explain differential sensitivity to agonists, membrane permeability kinetics, and blebbing displayed by receptors expressed in different cell types. For the human receptor, a prevalent C-terminal truncated splice variant, P2X7B, has been described, which fails to trigger membrane permeabilization to large molecules (Adinolfi et al., 2010; Cheewatrakoolpong et al., 2005). While its association with bone remodelling is still controversial, data from the present study encourage further investigation, as truncated splice variants of the P2X7 receptor in MSCs might serve as fine descriptors of risk for bone mass loss and osteoporotic fractures, in parallel to the association described for single-nucleotide polymorphisms of the P2X7 receptor gene.

Our findings clearly indicate that long-term activation of the P2X7 receptor with BzATP anticipates osteogenic differentiation and promotes mineralization of postmenopausal MSCs in culture. This was proven at early culture time points (*e.g.*, day 4 and 7), as increases in ALP activity and in the amount of osteogenic transcription factors, such as Runx-2 and Osterix, led to increased formation of mineralized bone nodules afterward (culture day 43). This correlates with data using newborn rats to study skeletal development (Panupinthu et al., 2008). Likewise, increases of Runx-2 expression and osteogenic differentiation of murine MSCs were observed subsequent to mechanical stimulation (*e.g.*, oscillatory fluid flow; Arnsdorf et al., 2009), *via* a RhoA-dependent mechanism involving deformation of the nuclear envelope and loosening of the chromatin. Participation of RhoA/ROCK and cytoskeletal dynamics also seems to be required for stretch-induced tenogenic differentiation of human MSCs (Xu et al., 2011). Evidence gathered in this study links P2X7 receptor-induced osteogenic differentiation and mineralization of postmenopausal MSCs to Rho associated kinase, since both phenomena were abrogated by preincubation with the highly potent and specific Rho kinase inhibitor, H1152 (*e.g.*, see Sasaki et al., 2002).

BzATP also promoted mineralization of cultured MSCs from young (14-yr-old) females through the P2X7 receptor/Rho-kinase pathway, although the effect in the younger group was much more exuberant than in postmenopausal women. Immunoreactivity against the P2X7 receptor was detected at early time points on MSCs from postmenopausal women, declining thereafter (7>21 days), whereas the opposite was found in MSCs from younger females (7<21 days) (Noronha-Matos et al., 2012). The same was verified regarding ATP-induced $[Ca^{2+}]_i$ transients (Noronha-Matos et al., 2012) and BzATP-evoked membrane blebbing (unpublished observations), which decreased significantly in postmenopausal MSCs from culture day 7 to 21. Differences in the P2X7 receptor expression and function might explain the changes in the mineralization potency of BzATP among the two age groups. One cannot, however, exclude the fact that the actions of adenine nucleotides are balanced through concurrent expression of specific NTPDases, namely NTPDase1, -2, and -3, whose levels raise in postmenopausal MSCs, as these cells differentiate (7<21 days; Noronha-Matos et al., 2012). Preliminary data from our group demonstrate that extracellular inactivation of BzATP is faster in more differentiated MSC populations. The regulatory potential of subtype specific NTPDases on P2X7 receptor commitment of MSCs to osteogenic differentiation deserves attention in future studies (see paper 3 below). Despite this constraint, we were able to show that activation of the P2X7–Rho-kinase axis enables MSCs from postmenopausal women to differentiate into osteoblasts, resuming mineralization to levels observed in young females.

Controlled ATP release from human osteoblasts was first described by Romanello *et al*. (Romanello et al., 2001), and since then, it has been a widely investigated area. Evidence now suggests that stimuli inducing bone formation and accelerated fracture healing (like mechanical loading, ultrasound, $1,25(OH)_2$ vitamin D_3, and bisphosphonates) promote ATP release from osteoblast-like cells (Alvarenga et al., 2010; Biswas and Zanello, 2009; Hayton et al., 2005; Romanello et al., 2006; Rumney et al., 2012). Interestingly, the amount of ATP released from osteoblasts depends on their differentiation state, with mature bone-forming cells releasing up to 7-fold more than proliferative immature cells (Orriss et al., 2009).

Despite the controversy regarding the mechanisms controlling ATP release from osteoprogenitor cells, our data indicate that these provide enough ATP to activate P2X7 receptors in young women, but the P2X7 receptor tonus may be severely affected in postmenopausal MSC cultures.

In summary, this study provided valuable information concerning the role of the P2X7 receptor on osteogenic differentiation of MSCs from postmenopausal women, which, together with previous findings from our group, led us to propose that adenine and uracil nucleotides (Noronha-Matos et al., 2012) and their derivatives (such as adenosine; Costa et al., 2011) may act as potential targets for therapeutic management of bone disorders leading to increased bone loss, osteoporosis, and fracture risk.

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Paper 3

(Manuscript in preparation)

Inhibition of NTPDase3 on bone marrow-derived mesenchymal stem cells may be a novel therapeutic strategy to increase bone formation in postmenopausal women

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ABSTRACT

The ability of bone marrow-derived mesenchymal stem cells (MSCs) to differentiate into bone-forming osteoblasts is compromised in postmenopausal women. Nucleotides, via several P2 purinoceptors, are important regulators of osteogenic differentiation of MSCs. The endogenous actions of nucleotides may be counteracted by breakdown through specific NTPDases. Here, we investigated the expression and function of specific NTPDases on osteogenic differentiation and mineralization of MSCs from young females and postmenopausal women. In the younger group, nucleotides progressively increased intracellular $[Ca^{2+}]_i$ and osteogenic differentiation of MSCs with the time in culture (7<21 days); parallel increases in alkaline phosphatase activity and in the expression of osteogenic transcription factors (Runx-2 and Osterix) and P2 purinoceptors (*e.g*, P2X7 and $P2Y_6$), were also observed. Conversely, we found that intracellular $[Ca^{2+}]_i$ and osteogenic differentiation markers became less evident with time (7<21 days) in cultures of MSCs from postmenopausal women. The extracellular hydrolysis of the nucleotides was faster in less proliferative and more differentiated cell populations, in agreement with increases in the expression of NTPDases1, -2 and -3 in differentiating postmenopausal MSCs (7<21 days). A major difference was found in the expression of NTPDase3 among MSCs from young females and postmenopausal women. Selective blockade of overexpressed NTPDase3 in postmenopausal MSCs with PSB 06126 (3 µM) or with the antibody hN3-$B3_s$ (0.5 µg/ml) led to increases in extracellular ATP levels and to osteogenic differentiation and mineralization of the cultures, via the activation of P2X7 and $P2Y_6$ receptors. Data suggest that inhibition of NTPDase3 on MSCs may be a novel therapeutic strategy to increase bone formation in postmenopausal women.

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (MSCs) present an extensive proliferative potential and are characterized by their ability to differentiate into various cell lineages (*e.g*., osteoblasts, adipocytes and chondrocytes). Among bone marrow stromal cells, mesenchymal stem cells (MSCs) are a rare population of non-haematopoietic stromal cells, which have the potential to differentiate into the osteoblast lineage, when incubated under proper stimuli (Bobis et al., 2006; Conget et al., 1999; Costa el al., 2011; Pittenger et al., 1999). However, their ability to differentiate into osteoblasts is compromised in postmenopausal women as compared to younger female patients (Noronha-Matos et al., 2012), leading to unbalanced bone resorption by osteoclasts.

Extracellular adenine nucleotides, like ATP, play important roles in the differentiation and function of osteoblasts. Both MSCs and differentiated osteoblasts constitutively release ATP when submitted to mechanical stress like weight bearing load, ultrasound stimulation or under pathological conditions, such as hypoxia and inflammation (Alvarenga et al., 2010; Brandao-Burch et al., 2012; Orriss et al., 2009). Their specific targets include several subtypes of P2Y (G-protein coupled) and P2X (ligand-gated ion channels) purinoceptors (Romanello et al., 2005). We provided evidence showing that, besides ATP, uracil nucleotides are also important regulators of osteogenic differentiation of MSCs through the activation of UDP-sensitive $P2Y_6$ receptors (Noronha-Matos et al., 2012). This study also confirmed that the actions of adenine and uracil nucleotides were balanced through specific NTPDases, which regulate their extracellular breakdown and, thereby, determine whether osteoblast progenitors are driven into proliferation or differentiation. Our findings showed that MSCs from postmenopausal women express ecto-NTPDase1 and -3 at all differentiation time points, whereas NTPDase2 only becomes evident in less proliferative and more differentiated cells.

Regarding the substrate preference, human NTPDase1 (also called CD39 or apyrase, EC 3.6.1.5) dephosphorylates ATP directly into AMP, with little accumulation of ADP. NTPDase2 (CD39LI, EC 3.6.1.3) is a preferential nucleoside

triphosphatase, leading to minimal accumulation of monophosphates, because it hydrolyses diphosphates 10-15 less efficiently than triphosphates (Kukulsky et al., 2005). NTPDase3 (CD39L3 or HB6) and NTPDase8 (hepatic ATPDase) are functional intermediates between NTPDase1 and NTPDase2 (Kukulsky et al., 2005). With the exception of NTPDase8, the apparent K_m values of human NTPDases for adenine nucleotides as substrates are in the low micromolar range; higher K_m values are obtained for the hydrolysis of uracil nucleotides, which may influence the relative potency of UTP/UDP compared to ATP/ADP. Among human MSCs, osteoprogenitors are distinguished because they express high amounts (>95%) of ecto-5'-nucleotidase/CD73 (Liu et al., 2009; see also Noronha-Matos et al., 2012), the rate limiting enzyme for adenosine formation from the catabolism of relased adenine nucleotides (Costa et al., 2011). Adenosine is also an important regulator of osteogenic differentiation of human MSCs through the activation of A_1, A_{2A} and A_{2B} receptors depending on the proliferation status of the cells (Costa et al., 2011). Because of their involvement in physiological processes, namely blood clotting, vascular inflammation, immune reactions and certain types of cancer, NTPDases are now considered as potential drug targets (Gendron et al., 2002).

Taking into consideration our findings on the kinetics of the extracellular catabolism of adenine/uracil nucleotides and adenosine formation via ecto-nucleotidases in human MSCs from postmenopausal women (Costa et al., 2011; Noronha-Matos et al., 2012), we hypothesized that differential expression and/or activity of specific NTPDases could contribute to the differences in osteogenic differentiation detected between young females and postmenopausal women. Membrane compartmentalization of ecto-enzymes, nucleotide releasing sites and purinoceptors has been demonstrated (reviewed by Yegutkin, 2008). This feature emphasizes the value of gathering information concerning extracellular metabolism and subsequent delivery of nucleotides to specific P1 and P2 purninoceptors in order to antecipate their pathophysiological relevance on osteogenesis, prompting for new pharmacological targets to control bone loss. Therefore, we consider worth to investigate the expression of NTPDase1, -2 and -3 and ecto-5'-nucleotidase along with certain P2 purinoceptors subtypes, by

immunofluorescence confocal microscopy in cultured MSCs derived from the bone marrow of thirteen postmenopausal women (56-83 years old) undergoing total hip arthroplasty and nine younger females (13-40 years old). We also studied the variations of extracellular ATP accumulation and breakdown of adenine and uracil nucleotides during osteogenic differentiation of MSCs from both age groups. The effects of selective P2 purinoceptor agonists and NTPDase inhibitors on osteogenic differentiation and mineralization of MSCs from postmenopausal women as compared to younger females were inferred by changes in (1) alkaline phosphatise (ALP) activity, (2) the expression of osteogenic transcription factors, Runx-2 and Osterix, and (2) calcium deposition in mineralized bone nodules (Alizarin Red staining). The ability of ATP, UTP and UDP to trigger intracellular $[Ca^{2+}]_i$ transients with respect to the growth state of primary MSCs was evaluated by confocal microscopy using the fluorescent Ca^{2+} indicator, Fluo4-NW.

MATERIALS AND METHODS

Reagents and antibodies

Adenosine (ADO), ADP, ATP, 3,4-dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt (Alizarin Red Staining), apyrase (Grade VI from potato, EC 3.6.1.5), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 4-nitrophenyl phosphate, uridine, UDP and UTP were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-[[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079), 6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine-5′-triphosphate trisodium salt hydrate (ARL 67156), N,N''-1,4-butanediyl-bis-[N'-(3-isothiocyanatophenyl) thiourea (MRS 2578), 1-Amino–4-(1-naphthyl) aminoanthraquinone-2-sulfonic acid sodium salt (PSB 06126) were obtained from Tocris Cookson Inc. (Bristol, UK). 2-Methoxy-6-chloro-9-(4-bis[β-chloroethyl] amino-1-methylbutylamino) acridine dihydrochloride (quinacrine mustard dihydrochloride) was obtained from Fluka (Buchs, Switzerland). The ATP Bioluminescence Assay Kit HS II was obtained from Roche Applied Science (Mannhelm, Germany). Dimethylsulphoxide (DMSO) and Folin phenol reagent

were from Merck (Darmstadt, Germany). Tetrabutylammonium was obtained from Waters Corporation (Milford, MA, USA).

All primary antibodies used in this study have previously been validated: anti-$P2Y_1$, anti-Runx-2 (M-70) and anti-Osterix (M-15) were from Santa Cruz (Santa Cruz, CA); anti-$P2Y_2$, anti-$P2Y_4$, anti-β-Actin and horseradish-peroxidase-conjugated secondary antibodies were from AbCam (Cambridge, UK); anti-$P2Y_6$ and anti-P2X7 were purchased from Alomone (Jerusalem, Israel); anti-osteocalcin and anti-Type I collagen were from AbD Serotec (Kidlington, Oxford, UK). The development and specificity of anti-human NTPDase2 and NTPDase3 has been reported previously (Dranoff et al., 2004; Munkonda et al., 2009). Genetic immunization protocol was carried out with plasmids (pcDNA3 for human NTPDase1 and pcDNA3.1 for human CD73) encoding each protein using New Zealand rabbits for antibodies against human NTPDase1 and Hartley guinea pigs for human CD73 antibodies; Hartley guinea pigs and New Zealand rabbits were obtained from Charles River Laboratories (Quebec City, Canada). NTPDase and CD73 antibodies were developed in the Centre de Recherche en Rhumatologie et Immunologie, University of Laval, Québec, QC, Canada (see http://ectonucleotidases-ab.com for further details). Alexa Fluor 488-labeled anti-rabbit, Alexa Fluor 568-labeled anti-mouse, Alexa Fluor 653-labeled anti-goat and the fluorescent calcium indicator Fluo-4NW were supplied by Molecular Probes (Invitrogen, Carlsbad, CA).

Cell cultures and phenotypic characterization

Human bone marrow samples were obtained from the neck of the femur of consecutive female patients (56-83 years old, *n*=13) undergoing total hip arthroplasty as a result of primary osteoarthrosis, and from younger female patients (13-40 years old, *n*=9) requiring bone engraftment for spinal fusion to correct scoliosis or fracture osteosynthesis. Handling of bone marrow samples and culture of adherent cells was performed during 10-15 days (until near confluence), as previously described (Noronha-Matos et al., 2012; 2014). First subcultures were maintained for 43 days in standard culture medium [α-minimal essential

medium (α-MEM) plus 10% foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B] supplemented with 50 µg/mL ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone to promote osteogenic differentiation. Bone marrow-derived mesenchymal stem cells (MSCs) cell cultures were established for 35 to 43 days in the absence (control) or in the presence of the test drugs that were added to the culture medium at day 1 (Costa el al., 2011; Noronha-Matos et al., 2012). Drugs were renewed in the culture at each medium change, *i.e.* twice a week.

Phenotypic characterization of the cells (first subculture) was performed by flow cytometry (Noronha-Matos et al., 2012). These cells exhibited positive immunoreactivity against CD105 (SH2), CD29 (integrin ß1) and CD117 (tyrosine-protein kinase Kit), which have been identified as surface markers of bone marrow-derived MSCs (Baddoo et al., 2003; Bobis et al., 2006; Boiret et al., 2005; Cognet and Minguell, 1999; Dennis et al., 2002; Gronthos et al., 2003; Pittenger et al., 1999). Conversely, the cells were negative for haematopoietic surface markers, like CD14 and CD45, which have been extensively used as a good argument to distinguish bone marrow haematopoietic cells from mesenchymal stem cells (Baddoo et al., 2003; Pittenger et al., 1999). Thus, first passage plastic-adherent human bone marrow cells obtained under the present experimental conditions are highly enriched in multipotent MSCs.

Viability/proliferation and osteogenic differentiation of bone marrow MSCs

Cell viability/proliferation was evaluated by the MTT assay (Costa el al., 2011; Noronha-Matos et al., 2012; 2014). Data from the MTT assay correlates positively with the results measuring cell proliferation from total DNA quantification per culture well (see *e.g.* Noronha-Matos et al., 2012). Osteogenic differentiation of bone marrow-derived MSCs was inferred as increases in alkaline phosphatase (ALP) activity and in the expression of osteogenic transcription factors, namely Runx-2 and Osterix. ALP activity was determined in cell lysates by colorimetric determination of *p*-nitrophenyl phosphate (PNP) hydrolysis, as described previously (Costa et al., 2011; Hoemann et al., 2009; Noronha-Matos et al., 2012;

2014); obtained values were expressed in nmol of PNP per minute normalized by the MTT absorbance (nmol min^{-1} MTT^{-1}) (Noronha-Matos et al., 2012). Levels of Runx-2 and Osterix proteins were determined by Western blot analysis at culture days 7 and 21 (Noronha-Matos et al., 2014). Equal protein amounts (10 µg) loaded into SDS-PAGE (10%) gels were transferred onto a polyvinylidenifluoride membrane using a Mini-Protean Tetra Cell coupled to a Mini-Trans-Blot module (Bio-Rad, Hercules, CA, USA). Blocked membranes were incubated with anti-human primary antibodies [1:200: anti-Runx-2 (M-70, rabbit), anti-Osterix (M-15, goat)]. β-Actin (rabbit) was used as control. The peroxidase detection system (1.25 mM luminol, 0.2 mM coumaric acid, 0.1M Tris pH 8.5, 0.032% hydrogen peroxide) was used for visualization of the immunoreactivity. Gels were analysed using a gel blot imaging system (ChemiDoc MP, Bio-Rad, Hercules, CA, USA). At culture day 43, calcium deposition in mineralized nodules was revealed by the Alizarin Red staining and photographed using a microscope (Zeiss Axiophot, Oberkochen, Germany) equipped with CCD camera (CoolSnap HQ, Ropers Inc., Tucson, AZ, USA) running an image acquisition software (MetaFluor 6.3, Photometrics, Tuczon, AZ, USA) (Costa el al., 2011; Noronha-Matos et al., 2014). Images were exported to Image J 1.37c software (NIH, Bethesda, MD, USA) for quantification of the area of bone-nodules. Results were expressed in total mineralized area per μm^2 and total number of bone nodules per culture well (Brandao-Burch et al., 2005; Noronha-Matos et al., 2012; 2014).

Single-cell $[Ca^{2+}]_i$ transients by confocal microscopy

Primary human MSCs were seeded into 35 mm dishes at a density of 2×10^4 cells/mL and allowed to grow for 7 and 21 days in supplemented α-MEM medium. Intracellular $[Ca^{2+}]_i$ oscillations were evaluated in cells loaded with the fluorescent Ca^{2+} indicator, Fluo-4NW (2.5 µM), as previously described (Noronha-Matos et al., 2012; 2014). Briefly, culture dishes were mounted on the stage of a laser-scanning confocal microscope (Olympus FV1000, Tokyo, Japan) and perfused continuously (1 mL/min) with gassed (95% O_2 + 5% CO_2) Tyrode's solution (pH=7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and

glucose 11.2, at 37ºC. Test drugs were delivered using a multichannel perfusion system (ValveLink8.2; Digitimer, Welwyn Garden City, UK). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by the Ca^{2+} ionophore, ionomycin (5 μM, 100% response) (Henriksen et al., 2006; Noronha-Matos et al., 2012; 2014).

Immunofluorescence staining

Human bone marrow-derived MSCs were allowed to grow in chamber slides for 7 or 21 days. Paraformaldehyde fixed cells were incubated, in the dark for 2 hours, with the following primary antibodies: NTPDase1 1:150 (hN1-9LI4, rabbit), NTPDase2 1:200 (hN2-Kw3I4, rabbit), NTPDase3 1:200 (hN3-B3S, mouse), ecto-5'-nucleotidase 1:300 (h50NT-2CI4, guinea-pig), $P2Y_2$ 1:150 (rabbit), $P2Y_4$ 1:75 (rabbit), $P2Y_6$ 1:75 (rabbit), P2X7 1:75 (rabbit), osteocalcin 1:75 (rabbit), and collagen type I 1:50 (rabbit). Alexa Fluor 488 (anti-rabbit), Alexa Fluor 568 (anti-mouse) and Alexa-Fluor 649 (anti-guinea pig) were applied as secondary antibodies for 1 h in the dark. Glass slides were mounted with VectaShield medium and stored at 4ºC. Observations were performed and analysed with a laser-scanning confocal microscope (Olympus FV1000, Tokyo, Japan) (Alqallaf et al., 2009; Costa et al., 2011; Noronha-Matos et al., 2012; 2014).

Kinetic experiments and HPLC analysis

The kinetics of inactivation of ATP, UTP and UDP in human primary MSC cultures was studied at days 7 and 21, at 37ºC (three replicas were performed in each individual experiment) (see *e.g.*, Costa et al., 2011; Noronha-Matos et al., 2012). After a 30-min equilibration period, cells were incubated with 100 μM of ATP, UTP, or UDP added to the culture medium in the conditions referred above (zero time). Samples (75 μl) were collected from each well at different times up to 30 min for high-performance liquid chromatography (HPLC, LaChrom Elite, Merck, Frankfurt, Germany) analysis of the variation of substrate disappearance and product formation. Aliquots of 20 μl of collected samples were used for nucleotide analysis. Concentrations of the substrates were plotted as a function of time

(progress curves). Half-degradation times of ATP, UTP, or UDP were calculated as previously described (Costa et al., 2011, Noronha-Matos et al., 2012). The enzymatic activity was normalized to the amount of viable cells given by the MTT assay (see *e.g.*, Noronha-Matos et al., 2012). The spontaneous degradation of adenine and uracil nucleotides at 37ºC in the absence of the cells was negligible over 30 min. At the end of the experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity (Keiding et al., 1974). The negligible activity of LDH in the samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental period.

Intracellular ATP staining with quinacrine

Quinacrine is a weak-base acridine derivative that binds ATP with high affinity and specificity. When excited by light at 476 nm it emits fluorescence in the 500-540 nm range and it has been widely used to visualize ATP-containing subcellular compartments in living cells (Irvin and Irvin, 1954; Orriss et al., 2009). MSCs were seeded on 35 mm glass bottom dishes (FluoroDish®, World Precision Instruments), at a density of $2x10^4$ cells/mL, and maintained in culture for 7 days. On the day of the experiment, cultured cells were washed twice with PBS before incubation with 30 μM quinacrine for 1 h, at 37ºC; cells were washed twice more and 150 μL of PBS were added per culture dish. Culture dishes were then mounted on a thermostatized perfusion chamber at the stage of an inverted laser-scanning confocal microscope (Olympus FV1000, Tokyo, Japan) equipped with a 20× magnification objective lens (LUCPLFL 20xPH; N.A. 0.45). Quinacrine fluorescence was detected in the time-lapse mode with FluoView Advanced Software (Olympus). Quinacrine was excited with the 488 nm Multi-line Ar laser. The emitted fluorescence was detected at 510-560 nm using the scanner of the confocal microscope (Olympus FV1000, Tokyo, Japan) (Brandao-Burch et al., 2012; Pinheiro et al., 2013).

Extracellular ATP quantification by bioluminescence

Extracellular ATP was detected with the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana, USA) using a multi detection microplate reader (Synergy HT, BioTek Instruments, Vermont, USA), as described elsewhere (Pinheiro et al., 2013). Briefly, cells were seeded into 96-well microplates, at $2x10^4$ cells/mL density, for 7 or 21 days (three replicas were performed in each individual experiment). At the beginning of the experiment, cells were washed twice with Tyrode's solution and allowed to equilibrate for 30 min, at 37ºC. After equilibrium, the cells were washed again and incubated with Tyrode's solution in the absence or in the presence of test drugs. After a 30-min time period, the incubation fluid was removed and snap-frozen in liquid nitrogen. Before adding the luciferin-luciferase mixture, collected samples were defrosted till 25ºC according to the manufacturer instructions. Sample bioluminescence was compared to external ATP standards prepared daily within the same concentration range; all samples were run in duplicate. Results are expressed as pmol of ATP per 30 min per well. The remaining incubation medium was used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity (Keiding et al., 1974), in order to evaluate cell integrity during the experimental period (see *e.g.* Pinheiro et al., 2013).

Presentation of data and statistical analysis

Results presented in this study are from bone marrow samples obtained from thirteen postmenopausal women (56 - 83 years old, n=13) and from nine younger females (13-40 years old, n=9). For each experiment and assay, 3-8 replicas were accomplished. Data are expressed as mean ± S.E.M. from an n number of individual experiments. Data from different individuals (from the same age group) were evaluated using one-way analysis of variance (ANOVA) and no significant differences in the pattern of cell behaviour were found. Statistical differences found between control and drug-treated cultures were determined by Bonferroni´s method. Values of $P<0.05$ were considered to represent significant differences.

Declaration of ethical approval

Informed consent to use the biological material, that would be otherwise discarded, was obtained. All procedures were approved by the Ethics Committees of Centro Hospitalar de Vila Nova de Gaia – Espinho (University Hospital), Gabinete Coordenador de Investigação / DEFI – Centro Hospitalar do Porto (CHP) and of Instituto de Ciências Biomédicas Abel Salazar (Medical School) of the University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki.

RESULTS

Osteogenesis is compromised in MSC cultures from postmenopausal women

Human MSCs (first subculture) from young females (13-40 years) and postmenopausal women (56-79 years) were allowed to grow for 28 days in an osteoblast-inducing medium. During this period, cell cultures were characterized for osteogenic differentiation measured as increases in ALP activity (Figure 21A) and in the expression of osteogenic transcription factors, such as Runx-2 and Osterix (Figure 21B and 21C). Calcium deposits corresponding to areas of mineralization of the extracellular matrix were evaluated by the Alizarin red histochemical staining at culture day 43 (Figure 21D).

Cell cultures from the younger group (13-40 years, *n*=9) exhibited much higher ALP activity than the postmenopausal group (56-79 years, *n*=9) (Figure 21A). The divergence in ALP activity found between the two age groups do not reflect differences in cell proliferation, since cell growth rate profiles of patients with different ages were very similar (see *e.g*. Noronha-Matos et al*.*, 2012).

Total cell lysates from MSC cultures of the two age groups were collected for Runx-2 and Osterix protein quantification by Western blot analysis at culture day 21. The typical Western blot gels depicted in Figure 21 show that Runx-2 and osterix proteins were less abundant in postmenopausal women (60-83 years) as compared to younger females (31-39 years) (Figure 21B and 21C). This difference

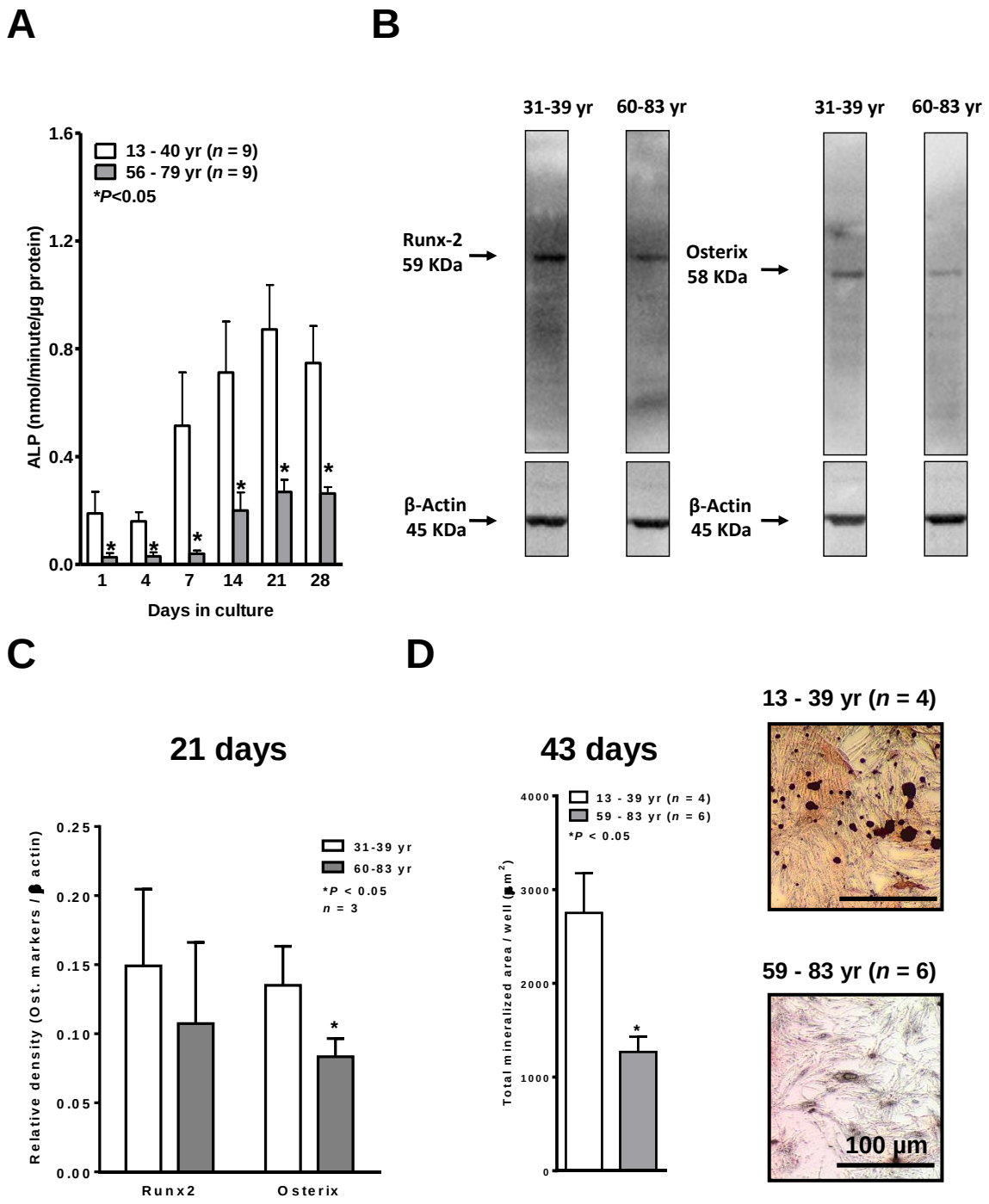

Figure 21. Osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs, first subculture) grown in an osteoblastic-inducing medium during 43 days. Bone marrow specimens were obtained from female patients with 56-79 years (postmenopausal group, *n*=9, gray bars) and 13-40 years (control group, *n*=9, white bars) (see Materials and Methods section).

Figure 21. *(Continued)* Panel (A) represents the activity of alkaline phosphatase (ALP) in cell lysates; results are expressed in nanomoles of *p*-nitrophenol produced per min per µg of total protein at certain time points. Panel (B) shows typical Western blot gels for Runx-2 (59 KDa) and Osterix (58 KDa) transcription factors in MSCs from young females (31-39 years) and postmenopausal women (60-83 years) at culture day 21; β-Actin (45 KDa) was used as an internal control. Panel (C), relative expression of Runx-2 and Osterix as a percentage of control detected in young females and postmenopausal women. Panel (D) represents matrix mineralization of MSC cultures (first subculture) at day 43. Bone marrow samples were obtained from female patients with 59-83 years (postmenopausal group, *n*=6, gray bar) and 13-39 years (control group, *n*=4, white bar). Calcium deposition was assessed by the Alizarin red test; typical micrographs evidencing bone nodules formation (red-brownish spots) are shown in the two right hand side panels (scale bar is 100 µm). Results are expressed as total mineralized area per culture well in μm^2. Bar graphs represent mean ± S.E.M. **P*<0.05 represent significant differences between the two age groups.

(28 and 38% for Runx-2 and Osterix, respectively) reached statistical significance (*P*<0.05) in the case of the Osterix. Decreases in the expression of Runx-2 and osterix osteogenic transcription factors may explain the reduction of ALP activity measured in postmenopausal MSC cultures (Ducy et al., 1997; Franceschi and Xiao, 2003; Kern et al., 2001; Nakashima et al., 2002; Paredes et al., 2004; Selvamurugan et al., 1998) (see Figure 21A).

The ability to mineralize the extracellular matrix, via ALP activity, measured as the total mineralized area per culture well given by the Alizarin red histochemical staining at day 43, was reduced (*P*<0.05) in postmenopausal women (59-83 years, *n*=6) as compared to MSC cultures from younger females (13-39 years, *n*=4) (Figure 21D). Overall, these results show that the osteogenic commitment of MSCs from postmenopausal women is impaired as compared to that observed in cells from younger females.

Differences in the expression of ionotropic P2X7 and metabotropic $P2Y_{2-6}$ in MSCs from young females and postmenopausal women

MSCs were allowed to grow in chamber slices for 7 and 21 days in osteogenic-inducing medium. Figure 22A shows that, in younger females, the expression of osteogenic-promoting receptors, like P2X7 and $P2Y_6$, increase with the time (7<21 days) as cells differentiate in culture. On the contrary, immunoreactivity for $P2Y_2$ and $P2Y_4$ receptors decreased from day 7 onwards, becoming practically undetectable on culture day 21 (Figure 22A). This pattern changed dramatically in

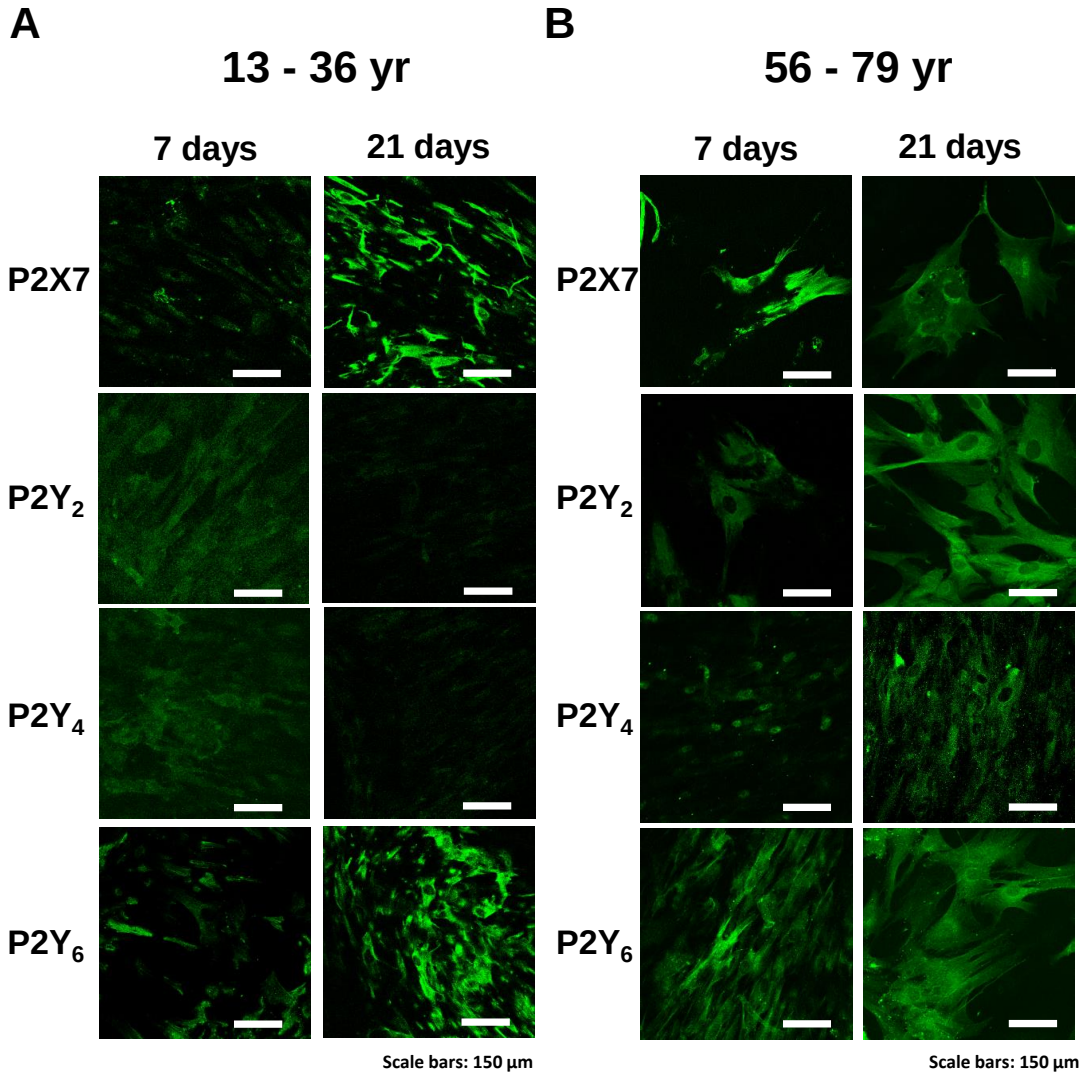

Figure 22. Immunocytochemical detection of P2X7, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on human primary bone marrow-derived mesenchymal stem cells (MSCs, 1st subculture) from female patients with **(A)** 13-36 years (control group, *n*=3) and **(B)** 56-79 years (postmenopausal group, *n*=5-6). Shown is the time-related immunoreactivity fluorescence detection by confocal microscopy of MSCs allowed to grow for 7 and 21 days in an osteoblast-inducing medium. Cells grown in eight-well chamber slides were processed for immunocytochemistry in parallel and were visualized keeping unaltered the settings of the confocal microscope throughout the procedure. For further details on immunofluorescence labelling see Materials and Methods section. Images are representative of three to five independent experiments. Scale bar is 150 µm.

MSCs from postmenopausal women (Figure 22B). Although the expression of P2X7 and $P2Y_6$ remained fairly constant as cultures progress (a slight decrease was observed in the case of the P2X7 receptor), the immunoreactivity against $P2Y_2$ and $P2Y_4$ receptors was more evident on day 21 compared to culture day 7. The preferential expression of the ATP- and UTP-sensitive inhibitory $P2Y_2$ and

$P2Y_4$ receptors on differentiated postmenopausal MSCs resembles that observed in rat primary osteoblasts (Orriss et al., 2006).

Age-related changes in intracellular $[Ca^{2+}]_i$ signals caused by ATP and uracil nucleotides (UTP and UDP) in MSCs

Taking into consideration the variation of P2 purinoceptors expression along osteogenic differentiation of MSCs from both age groups, we tested whether these differences impacted on cells function. MSCs from young females (13-36 years) and postmenopausal women (56-79 years) cultured for 7 or 21 days were loaded with the intracellular fluorescence calcium indicator, Fluo4-NW, before being challenged with 100 µM ATP, UTP and UDP on the stage of the confocal microscope (Figure 23).

At culture day 7, all nucleotides tested significantly ($P<0.05$) increased $[Ca^{2+}]_i$ in MSCs from postmenopausal women, but these compounds were much less effective in the cells collected from younger females. Superfusion of 7-day MSC cultures from postmenopausal women with ATP (100 µM, Figure 23A) elicited a fast $[Ca^{2+}]_i$ rise, which typically peaked 40 sec after nucleotide addition and decayed back to baseline within 1-2 min; a second progressive, but of much lower amplitude, ATP-induced $[Ca^{2+}]_i$ rise was observed following the initial high-magnitude $[Ca^{2+}]_i$ transient (see also Noronha-Matos et al., 2012). Under similar experimental conditions, both UTP (100 µM, Figure 23B) and UDP (100 µM, Figure 23C) triggered sustained increases in $[Ca^{2+}]_i$ that typically peaked 2-3 min after application of uracil nucleotides and decayed back to baseline after drug washout. It is worth noting that UDP-induced $[Ca^{2+}]_i$ rise had a higher magnitude than that caused by UTP when both signals were calibrated for the maximum $[Ca^{2+}]_i$ load produced by ionomycin (5 µM).

In MSCs from postmenopausal women, intracellular $[Ca^{2+}]_i$ responses caused by ATP, UTP and UDP were strikingly decreased with time in culture (7>21 days) (*cf.* Noronha-Matos et al., 2012), yet the opposite was verified in cells from younger females (Figure 23). That is, more differentiated MSCs from postmenopausal women were less responsive to stimulation by ATP and uracil

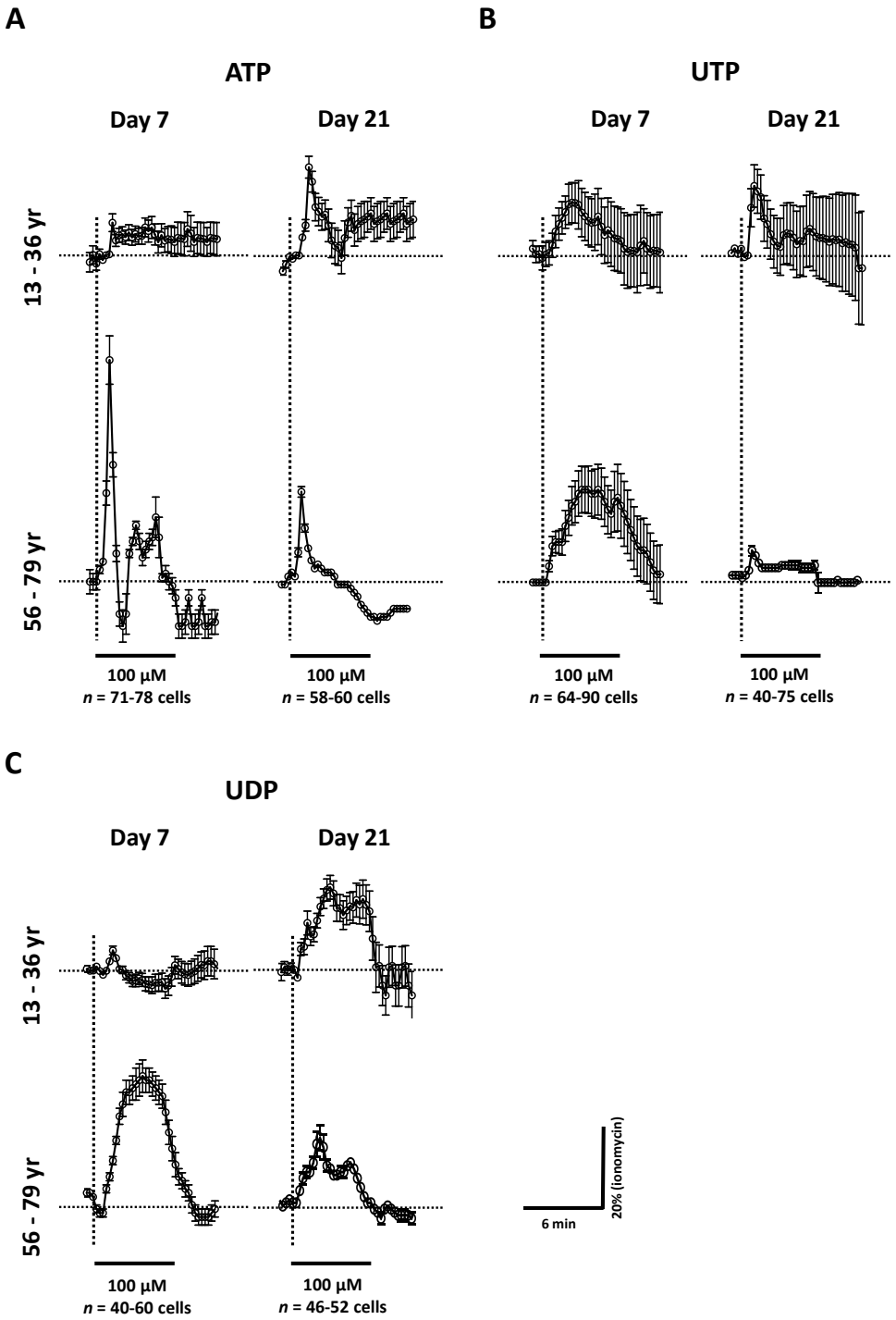

Figure 23. Effects of ATP, UTP and UDP (100 µM) on intracellular $[Ca^{2+}]_i$ oscillations in human primary bone marrow-derived mesenchymal stem cells (MSCs, 1st subculture) from female patients with 13-36 years (control group) and 56-79 years (postmenopausal group). Cells were allowed to grow in culture for 7 and 21 days in an osteoblast-inducing medium. Cells were loaded with the fluorescent calcium indicator, Fluo-4NW (2.5 µM, in PBS plus 2.5% pluronic acid) for 45 min at 37ºC.

Figure 23. *(Continued)* Changes in fluorescence were detected by laser-scanning confocal microscopy (Olympus FV1000, Tokyo, Japan) in the time-lapse mode. ATP (100 μM, A), UTP (100 μM, B) and UDP (100 μM, C) were applied during 6 min. Intracellular $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100 % response). The black bars at the bottom of each graph indicate the period of drug exposure. Each point represents pooled data from 3 (control group) and 6 (postmenopausal group) different individuals. The vertical bars represent S.E.M from an *n* number of evaluated cells.

nucleotides, despite the fact that these cells express higher amounts of P2 purinoceptors (see Figure 22B). In contrast to the divergence between nucleotide-induced $[Ca^{2+}]_i$ responses and P2 receptors expression in MSCs from postmenopausal women, we observed that $[Ca^{2+}]_i$ transients produced by 100 μM ATP, UTP and, in particular, UDP, corresponded to evident gains of expression of P2X7 and $P2Y_6$ receptors responding to ATP and UDP, respectively, when comparing 7-day and 21-day cultures from younger females.

Nucleotide-induced modifications in the osteogenic differentiation pattern of MSCs from young females and postmenopausal women

First passage MSCs from young females (14-16 years) and postmenopausal women (56-76 years) were cultured for 28 days in conditions that favour osteogenic differentiation in the absence and in the presence of 100 μM ATP, UTP and UDP. Continuous application of these nucleotides to the culture medium was devoid of effect on the proliferation of MSCs measured by the MTT assay (data not shown). Table 5 shows the variation of ALP activity of the cells exposed continuously to adenine and uracil nucleotides compared to the control cultures grown in the absence of test drugs.

In MSC cultures from postmenopausal women, ATP (100 μM) caused a biphasic effect decreasing ALP activity until a maximum was reached on culture day 14, returning back to baseline thereafter (Table 5). On the contrary, UTP (100 μM) and UDP (100 μM) significantly ($P<0.05$) increased the ALP activity of postmenopausal MSCs during the first week, but the effect of uracil nucleotides decreased progressively thereafter (Table 5). At days 21 and 28, the activity of ALP was about the same in the presence of UTP (100 μM) and UDP (100 μM), as compared to control cultures. The lack of effect on ALP activity contrast with the

Table 5. Variation of alkaline phosphatase (ALP) activity of human primary bone marrow-derived mesenchymal stem cells (MSCs, first subculture) from young females (14-16 years old) and postmenopausal women (59-76 years old) exposed continuously (during 28 days) to ATP (100 µM), UTP (100 µM) and UDP (100 µM).

		Days in culture			
	nmol/min/µg protein (Drug-Ctr)	7	14	21	28
ATP (100 µM)	14 - 16 yr	-0.12±0.07*	-0.16±0.04*	0.80±0.19*	-0.05±0.03
	59 -76 yr	0.00±0.01	-0.32±0.01*	-0.22±0.01*	-0.11±0.02*
UTP (100 µM)	14 - 16 yr	-0.02±0.04	-0.1±0.01*	0.02±0.11	0.24±0.08*
	59 -76 yr	0.13±0.04*	0.07±0.02*	0.01±0.03	0.01±0.01
UDP (100 µM)	14 - 16 yr	-0.04±0.06	-0.08±0.03	0.09±0.1	0.35±0.06*
	59 -76 yr	0.12±0.05*	0.08±0.03*	0.05±0.03	0.03±0.01

Values represent changes in the activity of ALP (nmol/min/µg protein) as compared to control cultures grown in the absence of test drugs at the same time points (see Figure 21A). Zero represents identity between the two values (drug vs. control); positive and negative values represent facilitation or inhibition of osteogenic cell differentiation relative to control data obtained at the same time points. Each value represents pooled data from three to six different individuals (Mean ± SEM); 6-8 replicas were performed for each individual experiment. *P<0.05 represents significant differences from control values obtained in the absence of test drugs.

increased expression of uracil nucleotide-sensitive $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors as cells differentiate (see Figure 22B).

At culture day 21, ATP (100 µM) increased, rather than decreased, ALP activity in MSCs from young female patients (14-16 years). Whilst UTP (100 µM) and UDP (100 µM) progressively increased the ALP activity as MSCs from the younger female group maturate (maximum ALP activity was reached on culture day 28), the differentiating effect of both uracil nucleotides decreased from the first week onwards in postmenopausal MSC cultures. The timing of the osteogenic differentiation promotion caused by ATP, UTP and UDP on MSCs from young females coincides with the highest expression levels of P2X7 and $P2Y_6$ receptors (see Figure 22A), which activation is known to favour osteogenesis and bone formation in human osteoprogenitor cell cultures (Noronha-Matos et al., 2012; 2014).

Differences in NTPDase1, -2, -3 and ecto-5'-nucleotidase/CD73 immunoreactivity in the plasma membrane of MSCs from young females and postmenopausal women

P2 purinoceptors activation leading to osteogenic differentiation of human MSCs may be balanced through specific NTPDases whose expression varies along maturation of the cells (see *e.g.* Noronha-Matos et al., 2012). These findings may explain the disparity between subtype-specific P2 receptors expression in MSCs and the effects of adenine and uracil nucleotides on intracellular $[Ca^{2+}]_i$ and ALP activity at the same differentiation time points. Therefore, we allowed MSCs from young females (13-36 years) and postmenopausal women (59-76 years) to grow in chamber slides for 7 and 21 days in supplemented α-MEM before immulabelling the cells with specific primary antibodies against human NTPDase1, -2 and -3, and ecto-5'-nucleotidase/CD73. Fluorescence immunoreactivity against these enzymes shows a cytoplasmic/membrane-staining pattern that is typical for ecto-enzymes (Figure 24).

In MSCs from postmenopausal women, fluorescence immunoreactivity for NTPDase1, -2 and -3 increased with time (7<21 days in culture) (Figure 24B). Interestingly, the immunoreactivity against NTPDase2 was almost unapparent in MSCs cultured for 7-days, but it became stronger in more differentiated (21-day) cultures. Interestingly, we did not observe such maturation changes along the time of the cells in culture in the expression of NTPDase1 and -2 in MSCs from younger females (Figure 24A). We found a remarkable major difference concerning the expression of NTPDase3 among the cells from women of the two age groups, as this enzyme was totally absent in MSCs from younger females whilst its expression was increasingly more evident in maturating cells from postmenopausal women (Figure 24A).

The immunoreactivity against ecto-5'-nucleotidase/CD73, an enzyme that is typically expressed (>95%) by osteoprogenitor cells (see *e.g.* Lu et al., 2010), was detected at all maturation stages of MSCs from both young females and postmenopausal women. These findings suggest that adenosine formation, *via* ecto-5'-nucleotidase/CD73, does not account for the changes in the osteogenic

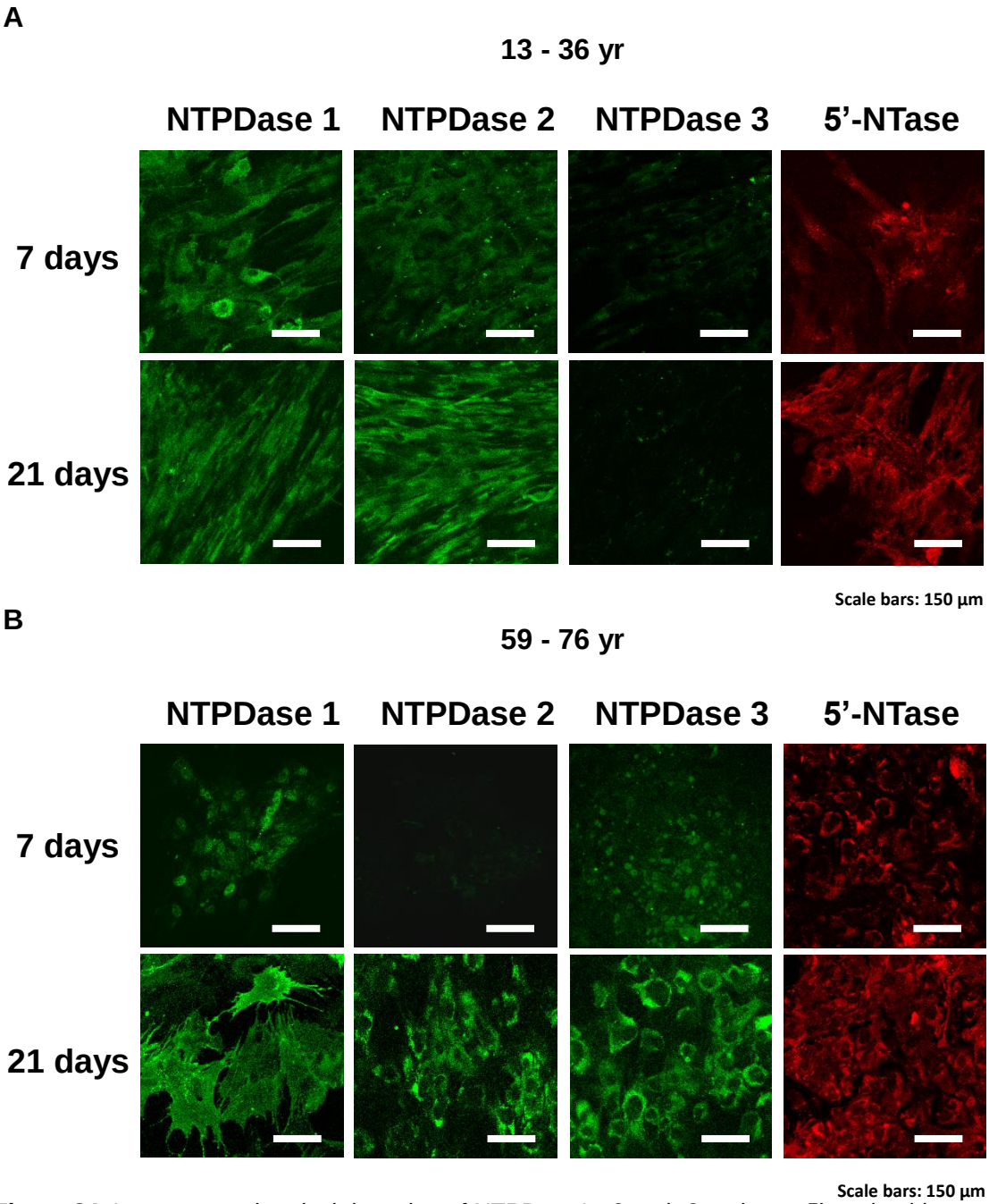

Figure 24. Immunocytochemical detection of NTPDase1, -2 and -3 and ecto-5'-nucleotidase on human primary bone marrow-derived mesenchymal stem cells (MSCs, 1st subculture) from female patients with **(A)** 13-36 years (control group) and **(B)** 59-76 years (postmenopausal group). Shown is the time-related immunoreactivity fluorescence detection by confocal microscopy of MSCs allowed to grow for 7 and 21 days in an osteoblast-inducing medium. Cells grown in eight-well chamber slides were processed for immunocytochemistry in parallel and were visualized keeping unaltered the settings of the confocal microscope throughout the procedure. For further details on immunofluorescence labelling see Materials and Methods section. Images are representative of three to five independent experiments. Scale bar is 150 µm.

differentiation potential of these cells in the two age groups, unless otherwise deficits occur upstream AMP substrate production from released adenine nucleotides.

Age related-changes in the pattern of the extracellular catabolism of ATP and uracil nucleotides (UTP and UDP) in cultured MSCs by HPLC

Figure 25 illustrates the time course of the extracellular catabolism of ATP and uracil nucleotides (UTP and UDP) in MSCs from young females (13-36 years) and postmenopausal women (59-76 years), which were allowed to grow in culture for 7 (Figure 25Ai, 25Bi and 25Ci) and 21 (Figure 25Aii, 25Bii and 25Cii) days in an osteogenic-inducing medium. Because enzymatic activity is usually represented as a function of the total protein content of the samples, yet in osteoblast differentiating cultures type I collagen accounts to 85-90% of the organic matrix, we decided to normalize the NTPDase activity by the amount of viable cells given by the MTT assay (Figure 25Aiii, Biii and Ciii) (*cf.* Noronha-Matos et al., 2012).

In MSCs from young females, ATP (100 μM) was hydrolysed with a half-degradation time of 121±71 min and 32±7 min at culture days 7 (Figure 25Ai) and 21 (Figure 25Aii), respectively; the half-degradation times calculated for postmenopausal MSCs were respectively 24±2 min and 12±1 min at culture days 7 (Figure 25Ai) and 21 (Figure 25Aii). Normalization of the ATP hydrolytic activity to the amount of viable MSCs from both age groups demonstrates that speed up of ATP (100 μM) catabolism as cultures progressed is due predominantly to the concurrent increase in the number of cells, since the net enzymatic activity decreased by 25-50% (Figure 25A). Comparing the normalized NTPDase activity of MSCs from both age groups, we show here that MSCs from postmenopausal women hydrolyse ATP (100 μM) 2.1 times faster than younger females, if one considers data obtained at culture day 21 (Figure 25Aiii).

Extracellular UTP (100 μM) was hydrolysed with half-degradation times of 124±27 and 34±5 min in MSCs from young females grown in culture for 7 (Figure 25Bi) and 21 (Figure 25Bii) days, respectively. In postmenopausal MSC cultures, UTP (100 μM) was hydrolysed with half-degradation times of 142±29 and 12±1

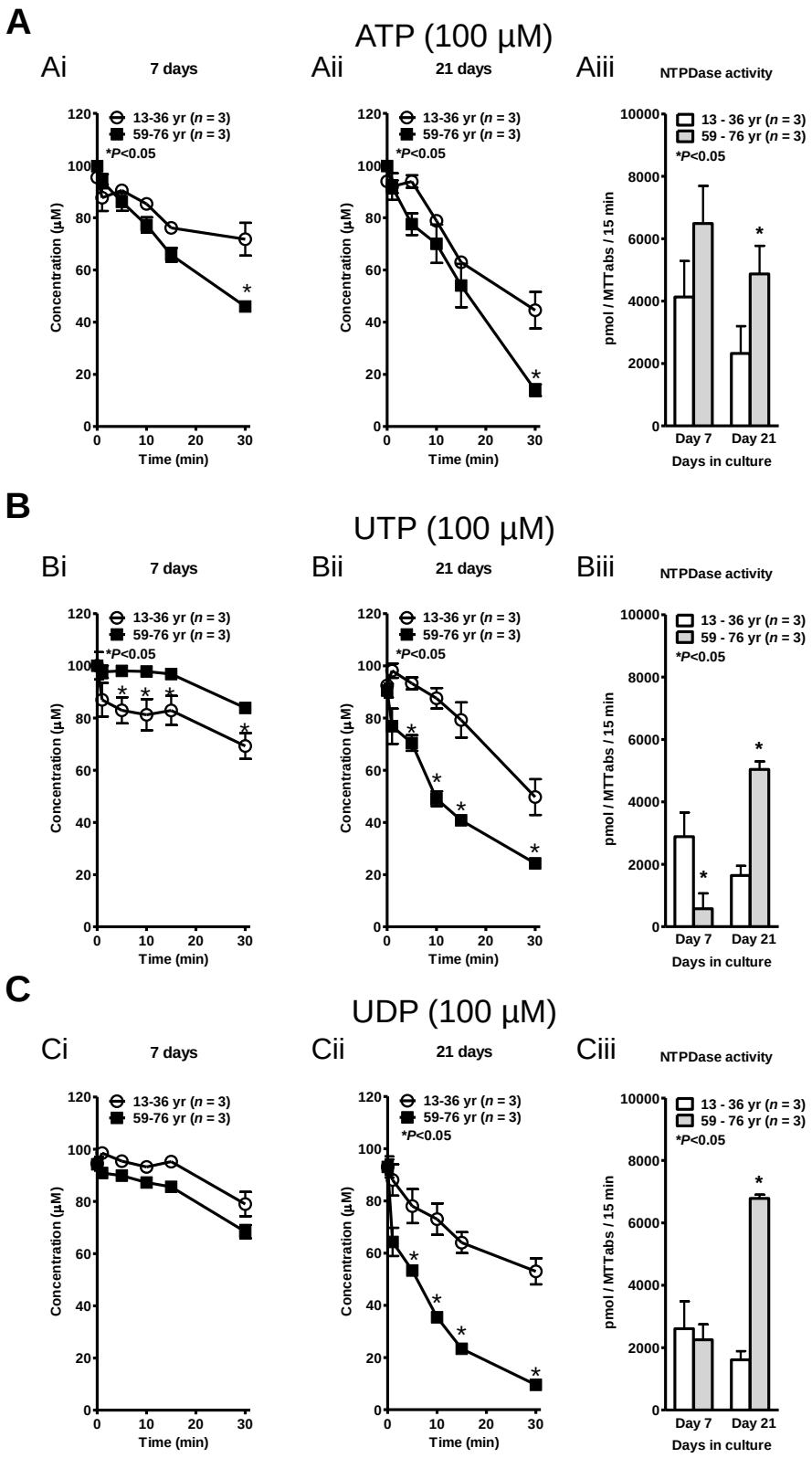

Figure 25. Time course of extracellular ATP (100 µM) **(A)**, UTP (100 µM) **(B)** and UDP (100 µM) **(C)** metabolism and total NTPDase activity in human primary bone marrow-derived

Figure 25. *(Continued)* mesenchymal stem cells (MSCs, 1st subculture) from female patients with 13-36 years (control group) and 59-76 years (postmenopausal group). Cells were allowed to grow for 7 (Ai, Bi, Ci) and 21 (Aii, Bii, Cii) days in an osteoblast-inducing medium. Nucleotides (100 µM) were added to the culture medium at zero time. Samples (75 µl) were collected from each well at the indicated times in the abscissa. Each collected sample was analyzed by HPLC to separate and quantify ATP, UTP or UDP in MSCs from young females (open circles) and postmenopausal women (filled squares). The NTPDase activity (Aiii, Biii, Ciii) was determined 15 minutes after exposure of the cells to the nucleotides; the activity of NTPDases was normalized by the amount of viable cells given by the MTT assay (control group, day 7: 0.35±0.05 A/cm^2 *vs* day 21: 1.08±0.20 A/cm^2, *n*=7; postmenopausal group, day 7: 0.48±0.02 A/cm^2 *vs* day 21: 0.89±0.05 A/cm^2, *n*=6). Each point represents pooled data from three individuals; two replicas were performed in each individual experiment. The vertical bars represent S.E.M. and are shown when they exceed the symbols in size. **P*<0.05 represent significant differences between the two age groups.

min at corresponding time periods, days 7 (Figure 25Bi) and 21 (Figure 25Bii), respectively. The progress curves of UDP (100 µM) disappearance in MSC cultures from young females and postmenopausal women at days 7 and 21 are shown in Figure 25C. The results show that extracellular UDP (100 µM) is slowly inactivated in less differentiated cells (Figure 25Ci), but the rate of UDP catabolism speeds up significantly (*P*<0.05) as cultures progressed to more differentiated status (Figure 25Cii). In MSCs from young females, UDP (100 µM) was metabolised with half-degradation times of 151±48 and 41±5 min at culture day 7 (Figure 25Ci) and 21 (Figure 25Cii), respectively, whereas in the postmenopausal group it was hydrolysed with half-degradation times of 69±16 and 6±1 min at corresponding time periods, days 7 (Figure 25Ci) and 21 (Figure 25Cii), respectively. The normalized NTPDase activity at culture day 21 shows that postmenopausal MSCs hydrolyse UTP (100 µM, Figure 25Biii) and UDP (100 µM, Figure 25Ciii) respectively 3 and 4.6 times faster than cultured cells from younger females.

The presence of a saturating concentration (10 mM) of β-glycerophosphate in the culture medium did not alter the degradation kinetics of ATP and uracil nucleotides (UTP and UDP), indicating that the contribution of non-specific phosphatases, namely ALP (EC 3.1.3.1) to the extracellular catabolism of nucleotides applied in a 100-µM concentration is negligible (data not shown).

Extracellular ATP accumulation in MSC cultures from young females and postmenopausal women

Human MSCs and osteoblast-like cells release ATP constitutively without cell damage (see *e.g.* Brandao-Burch et al., 2012; Buckley et al., 2003; Genetos et al., 2005; Riddle et al., 2007; Romanello et al., 2001). Given that maturation of metabolizing enzymes and purinoceptors may determine divergent effects of released nucleotides in differentiating cell populations, we designed experiments to measure the accumulation of ATP in the extracellular fluid collected from MSCs from young females (13-36 years) and postmenopausal women (59-82 years) grown in culture for 7 and 21 days in an osteogenic-inducing medium (Figure 26).

Quinacrine fluorescence cell staining has been widely used to visualize ATP-containing sub-cellular compartments in living cells (Irvin and Irvin, 1954; Orriss et al., 2009). Figure 26A shows that quinacrine (30 μM)-loaded MSCs exhibit a clear granular-vesicular staining pattern in the cytosol corresponding to intracellular ATP stores. Confirmation that MSCs constitutively release ATP was obtained measuring the nucleotide content in the fluid collected from MSCs cultures (Figure 26B). In 7-day cultures, accumulation of ATP in culture media during 30 min was about the same in samples collected from young females (0.24±0.04 pmol per well) and postmenopausal women (0.26±0.07 pmol per well). Whilst the ATP content of the samples decreased significantly (P<0.05) from culture day 7 to 21 in MSCs from young female patients (0.24±0.04 *vs* 0.11±0.06 pmol per well, respectively), the opposite was verified in cells isolated from postmenopausal women (0.26±0.07 *vs* 0.35±0.06 pmol per well, respectively) (Figure 26B). In order to confirm that these differences were not due to cell damage, we measured the lactate dehydrogenase (LDH) activity in the same samples. Cell membrane integrity was confirmed in all cell populations analysed because no significant (P<0.05) divergence was observed in LDH activity values (Figure 26B).

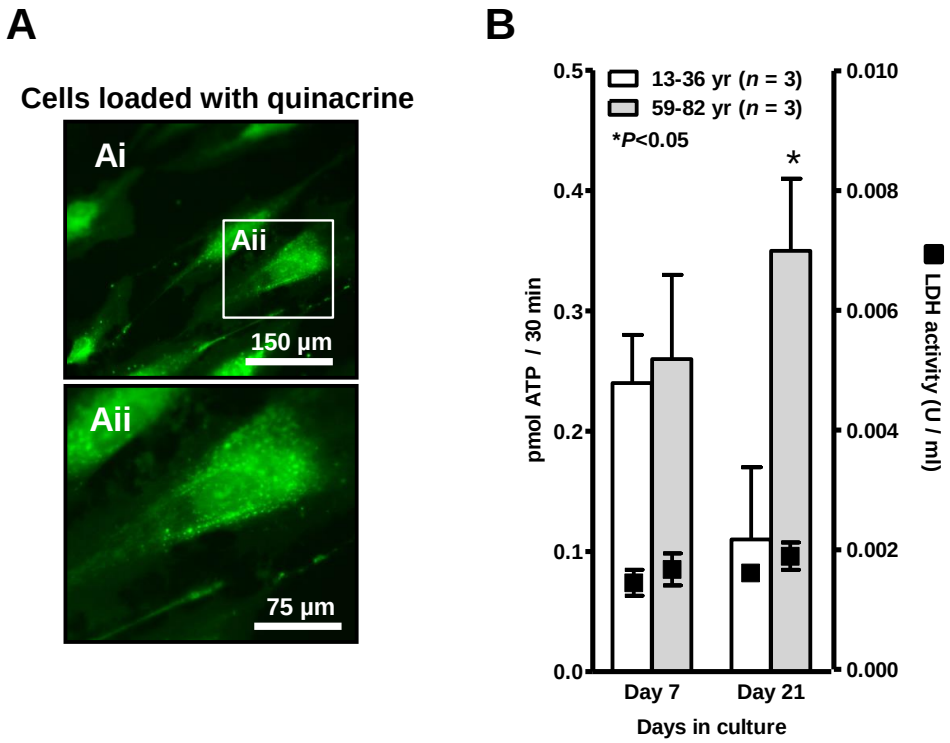

Figure 26. Constitutive ATP release from bone marrow-derived mesenchymal stem cells (MSCs, 1st subculture) from female patients with 13-36 years (control group) and 59-82 years (postmenopausal group). The cells were allowed to grow in an osteoblast-inducing medium. Panel (A) represents 7-day cells loaded with quinacrine exhibiting a clear granular-vesicular staining pattern in the cytosol corresponding to intracellular ATP stores (Ai, scale bar is 150 µm; Aii, scale bar is 75 µm). Panel (B), comparison of ATP accumulation in culture media of MSCs from young females (13-36 years) and postmenopausal women (59-82 years), which were allowed to grow for 7 and 21 days. Samples were collected 30 min after changing the culture medium. The ATP content of the samples was quantified by the luciferin-luciferase bioluminescence assay. In parallel, we measured the lactate dehydrogenase activity (LDH, filled squares) of the same samples to evaluate the integrity of the cells. Represented is pooled data from three individuals; two replicas were performed in each individual experiment. The vertical bars represent S.E.M. are shown when they exceed the symbols in size. *P<0.05 represent significant differences between the two age groups.

Inhibition of NTPDase3 favours extracellular ATP accumulation and promotes osteogenic differentiation and mineralization of MSCs from postmenopausal women

Considering that overexpression of NTPDase3 may account to faster nucleotide inactivation and to the partial loss of osteogenic potential of MSCs from

postmenopausal women compared to younger female patients, we decided to test whether selective inhibition of the activity of this enzyme could restore the osteogenic potential of postmenopausal MSCs. To this end we evaluated the effect of PSB 06126 (3 μM), an anthraquinone derivative that selectively inhibit NTPDase3 (Baqi et al., 2009) on osteogenic differentiation and mineralization of MSCs from postmenopausal women, which were cultured for 43 days in an osteogenic-inducing medium. Inhibition of NTPDase3 with PSB 06126 (3 μM) increased (by 3.6 fold, $P<0.05$) ATP accumulation in culture media of MSCs from postmenopausal women. On culture day 21, PSB 06126 (3 μM) significantly ($P<0.05$) increased (1) the ALP activity of MSCs by 43±17% above baseline (Figure 27A) and (2) the expression of Runx-2 by more than 5-fold the control level, without affecting downstream Osterix transcription factor (Figure 27B). Continuous application of the selective NTPDase3 inhibitor, PSB 06126 (3 μM), to the culture medium was devoid of effect on MSCs proliferation measured by the MTT assay. These findings contrast with those obtained with ARL 67156, a bromaminic acid derivative that preferentially inhibits NTPDase1 at 50 μM (Baqi et al., 2009), and sodium polyoxotungstate (POM-1, 10-100 μM), the non-selective inhibitor of NTPDases1, -2 and -3 (Wall et al., 2008), which decreased both MSCs proliferation and ALP activity (data not shown).

Figure 28 shows that selective inhibition of NTPDase3, either with PSB 06126 (3 μM) or with the monoclonal anti-human NTPDase3 antibody (hN3-B3$_S$, 0.5 μg/ml) (Munkonda et al., 2009), significantly ($P<0.05$) increased matrix mineralization of MSCs cultures from postmenopausal women at culture day 43. The mineralization effect caused by PSB 06126 (3 μM) and hN3-B3$_S$ (0.5 μg/ml) was prevented after pretreatment with A438079 (3 μM) and MRS 2578 (100 nM), which selectively antagonize osteogenic promoting P2X7 and P2Y$_6$ receptors, respectively (see *e.g.* Noronha-Matos et al., 2012; 2014; Panupinthu et al., 2008). At the concentrations used in this study, neither A438079 (3 μM) nor MRS 2578 (100 nM) modified the total mineralized area of the cultures when used alone, thus indicating that endogenously produced nucleotides may not reach concentrations high enough to activate P2X7 and P2Y$_6$ in postmenopausal MSCs unless

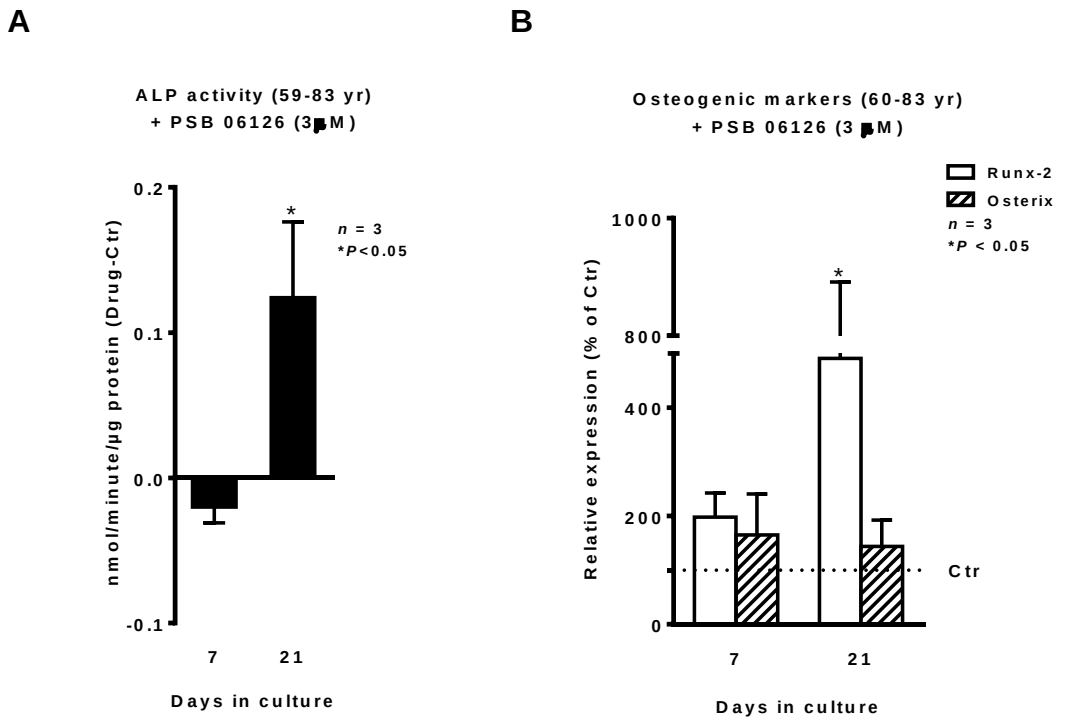

Figure 27. Selective inhibition of NTPDase3 with PSB 06126 increase osteogenic differentiation of postmenopausal bone marrow-derived mesenchymal stem cells (MSCs, 1st subculture) cultured for 28 days in an osteogenic-inducing medium. Panel (A), represented is the variation of alkaline phosphatase (ALP) activity of cultured MSCs exposed continuously to PSB 06126 (3 µM) as compared to control cultures grown in the absence of the NTPDase3 inhibitor (see Figure 21A). Zero represents identity between the two values (drug vs. control); positive and negative values represent facilitation or inhibition of osteogenic differentiation relative to control data obtained at the same time points. Each column represents pooled data from three individuals; 3-8 replicas were performed for each individual experiment. Panel (B), represented is the relative expression of the osteogenic transcription factors, Runx-2 (59 kDa) and Osterix (58 kDa), detected by Western blot analysis in MSCs from postmenopausal women (60-83 years) grown in culture for 7 and 21 days in the presence of PSB 06126 (3 µM) as compared to control values without adding the NTPDase3 inhibitor (dashed horizontal line). β-Actin (45 kDa) was used as control. Bars represent means ± S.E.M from three individuals. **P*<0.05 represent significant differences from control values obtained in the absence of PSB 06126.

NTPDase3 is inhibited. In contrast, apyrase (2.5 U/mL, EC 3.6.1.5, also called NTPDase1/CD39 or ATP diphosphohydrolase), the enzyme that catalyses inactivation of nucleoside triphosphates into their monophosphate derivatives with little or none accumulation of diphosphates, significantly (*P*<0.05) decreased bone nodule formation when applied alone and prevented PSB 06126 (3 µM)-induced mineralization of MSC cultures (Figure 28B). These results suggest that inhibition

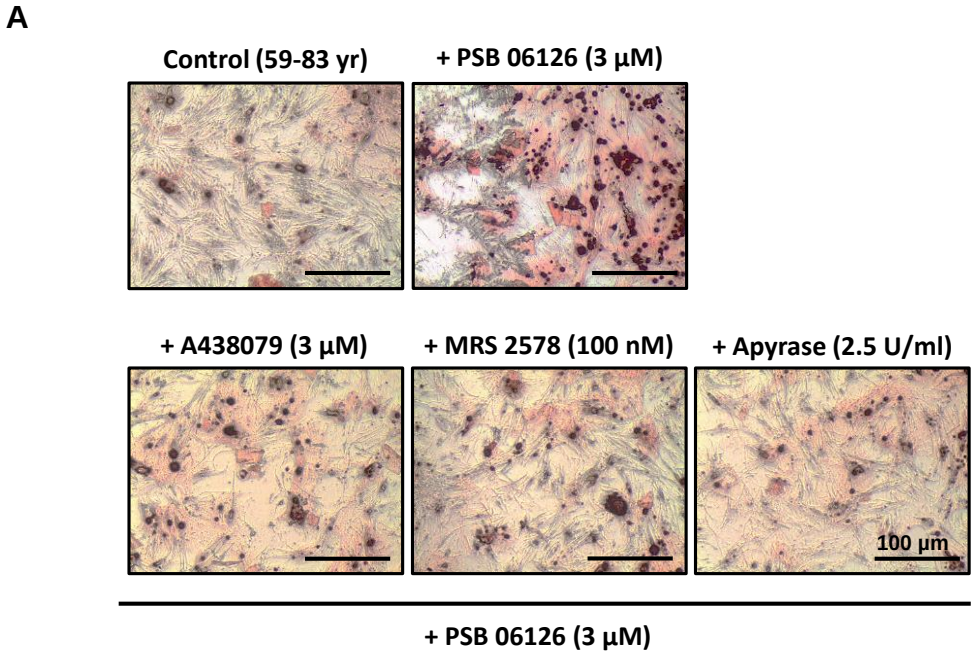

Figure 28. Selective inhibition of NTPDase3, either with PSB 06126 (3 µM) or with the monoclonal antibody against the human enzyme (hN3-B3S, 0.5 µg/ml), promotes mineralization of postmenopausal (59-83 years) bone marrow-derived mesenchymal stem cells (MSCs)

Figure 28. *(Continued)* in culture. Calcium deposition was assessed by the Alizarin red test. Panel (A), shows typical micrographs evidencing the formation of bone nodules (red-brownish spots) in MSC cultures exposed continuously for 43 days to PSB 06126 (3 µM) applied either alone or in the presence of A438079 (3 µM, a P2X7 selective antagonist), MRS 2578 (100 µM, a $P2Y_6$ selective antagonist), or apyrase (2.5 U/ml). Scale bar is 100 µm. Panel (B), results are expressed as total mineralized area per culture well. Each column represents pooled data from three to eight individuals; 3-6 replicas were performed for each individual experiment. The vertical bars represent S.E.M. *,**P<0.05 represent significant differences compared to control values (no drugs added) or with the effects of PSB 06126 or hN3-$B3_S$ applied alone, respectively.

of NTPDase3 activity with PSB 06126 (3 µM) or hN3-$B3_S$ (0.5 µg/ml) in postmenopausal MSCs recapitulates the mineralization potential of younger females (see Noronha-Matos et al., 2014), whose cells lack NTPDase3 (Figure 24A), an effect that requires the activation of P2X7 or $P2Y_6$ respectively by ATP and UDP. We also considered the use of siRNA to selectively prevent NTPDase3 expression in order to confirm the results obtained with PSB 06126 and hN3-B3s. Unfortunately maximum knockdown of a gene transcript usually occurs 48 hours after transfection. The duration of knockdown depends on a number of factors, including the transcription rate of the gene within the cell, but rarely lasts beyond 96 hours. Given that our study involves long term cultures, which go beyond 40 days, it is highly unlikely that full silencing of the gene transcript would occur under such experimental conditions.

DISCUSSION

This study was designed to investigate age-dependent changes in the "purinome" (Volonté and D'Ambrosi, 2009), comprising nucleotide release sites, extracellular metabolizing enzymes and purinoceptors expression/activation, in order to understand the mechanisms underlying impairment of osteogenic differentiation and matrix mineralization of primary cultures of MSCs from postmenopausal women as compared to younger females. Our results show that there is a notorious discrepancy between the expression time course and function of osteogenic-inducing P2 purinoceptors in differentiating MSCs from postmenopausal women. In contrast to younger females, nucleotide-induced intracellular $[Ca^{2+}]_i$ signals and osteogenic commitment of MSCs (measured as

ALP activity, expression of Runx-2 and Osterix transcription factors and matrix mineralization) declined along maturation of the cells in culture. The loss of purinergic signalling in postmenopausal MSCs is positively correlated with gains in the expression and activity of extracellular nucleotide metabolizing enzymes, in particular the NTPDase3; this enzyme is absent in cells from younger females but it becomes highly expressed in postmenopausal MSCs. Here, we provided the first evidence demonstrating that inhibition of NTPDase3, either with PSB 06126 (Baqi et al., 2009) or with a monoclonal antibody against the human enzyme (hN3-B3$_S$) (Munkonda et al., 2009), rescued the osteogenic potential of postmenopausal MSCs to levels observed in younger females by promoting the activation of P2X7 and $P2Y_6$ receptors by endogenous ATP and UDP, respectively. In view of this, we recommend inhibition of NTPDase3 as a novel therapeutic strategy to increase the osteogenic commitment of MSCs leading to bone formation in postmenopausal women.

Our data unequivocally indicate that osteogenic differentiation of MSCs is compromised in postmenopausal women as compared to younger females. This was inferred considering that postmenopausal MSCs present lower ALP activity and reduced levels of the osteogenic transcription factors, Runx-2 and Osterix (Ducy et al., 1997; Franceschi and Xiao, 2003; Kern et al., 2001; Nakashima et al., 2002; Paredes et al., 2004; Selvamurugan et al., 1998). This trait anticipates a reduced matrix mineralization capability that was confirmed in the postmenopausal group at later culture stages. In fact, previous studies using Runx-2-deficient mice revealed a complete absence of differentiated osteoblasts (leading to low ALP expression) and bone nodule formation (Komori et al., 1997; Otto et al., 1997). Likewise, mouse embryos lacking Osterix, a zinc-containing transcription factor essential for osteoblast differentiation and bone formation that acts downstream Runx-2, do not express osteoblast differentiation markers, like osteocalcin and alkaline phosphatase (ALP) (Nakashima et al., 2002). In addition, gene expression analysis revealed down-regulation of Runx-2 in MSCs from patients with osteoporosis (Dalle Carbonare et al., 2009).

Previous studies from our group demonstrated that postmenopausal MSCs in culture express ionotropic P2X7 receptors (Noronha-Matos et al., 2012). Our findings showed that immunoreactivity against P2X7 receptor was higher at early time points (culture day 7), but declined slightly thereafter (see also Figure 22). These findings are in agreement with the RT-PCR analysis performed by Orriss and col. (2006) in rat primary osteoblasts and suggest that P2 purinoceptors expression in osteoprogenitor cells is differentiation-dependent. We showed that activation of the P2X7 receptor favours osteogenic commitment of human MSCs and bone mineralization through a mechanism involving plasma membrane zeiosis and cytoskeleton rearrangements triggered by a Rho kinase-dependent pathway (Noronha-Matos et al., 2014). These findings fully support the increase in the postmenopausal fracture risk observed in women presenting loss of function polymorphisms of the P2X7 receptor (Jorgensen et al., 2012; Ohlendorff et al., 2007). Despite our findings proving that activation of the P2X7 receptor with BzATP promotes osteogenesis of MSCs derived both from young females and postmenopausal women (Noronha-Matos et al., 2014), endogenous ATP might not reach high enough levels to activate P2X7 receptors in MSCs from postmenopausal women unless cultures were treated with selective inhibitors of NTPDase3, like PSB 06126 (3 µM) or hN3-B3$_S$ (this study).

Besides the recognized role of ATP in bone remodelling via ionotropic P2X7 among other P2 receptors, we presented compelling evidences that uracil nucleotides facilitate osteogenic differentiation of postmenopausal MSCs predominantly through the activation of UDP-sensitive P2Y$_6$ receptor (Noronha-Matos et al., 2012). This was observed despite P2Y$_2$ and P2Y$_4$ receptors recognizing both ATP and UTP have also been localized in human bone-forming cells (Bowler et al., 1995; Maier et al., 1997), where they may be involved in the inhibition of bone mineralization particularly when the nucleotides were used in low concentrations (Hoebertz et al., 2002; Orriss et al., 2007). These intriguing observations, together with the lack of information regarding the relative abundance of each receptor subtype and their exact role during osteogenic differentiation of human osteoprogenitor cells, prompted us to investigate changes

in the expression of specific P2 purinoceptors with impact on bone formation in differentiating MSC cultures obtained from young females and postmenopausal women.

In younger females, the expression of osteogenic-promoting receptors, P2X7 and $P2Y_6$, progressively increase towards more differentiated cells, whereas $P2Y_2$ and $P2Y_4$ receptors become practically undetectable on culture day 21. Curiously, an opposite P2 receptors maturation pattern was observed in MSCs from postmenopausal women; whilst P2X7 and $P2Y_6$ receptors expression remained fairly constant as cultures progress, the immunoreactivity against $P2Y_2$ and $P2Y_4$ receptors became more evident in more mature cells. Differences in P2 receptors expression among postmenopausal and younger females, namely the growing expression of inhibitory ATP- and UTP-sensitive $P2Y_2$ and $P2Y_4$ receptors, may partially counteract the bone-promoting effect of P2X7 and $P2Y_6$ receptors (Hoebertz et al., 2002; Orriss et al., 2007). Our data also demonstrate that variation of P2 purinoceptors expression during maturation of MSCs impact on intracellular $[Ca^{2+}]_i$ signals and osteogenic differentiation of these cells. In contrast to MSCs from young females where appearance of osteogenic-promoting receptors, P2X7 and $P2Y_6$, positively correlates with the magnitude of intracellular $[Ca^{2+}]_i$ signals and ALP activity of the cultures, we observed an age-related divergence between P2 purinoceptors expression and osteogenic commitment induced by adenine and uracil nucleotides in MSCs from postmenopausal women leading to impairment of bone formation.

P2 purinoceptors activation leading to osteogenic differentiation of human MSCs may be balanced through specific nucleotide metabolizing enzymes (*e.g*. NTPDases) whose expression may also vary along maturation of the cells (Noronha-Matos et al*.*, 2012). This argument may explain the disparity between subtype-specific P2 receptors expression in MSCs and the effects of adenine and uracil nucleotides on intracellular $[Ca^{2+}]_i$ and ALP activity at the same differentiation time points. In contrast to data obtained regarding the ATP catabolism which largely depended on the amount of cells at a given stage, normalization of MSCs ability to hydrolyse uracil nucleotides (UTP and UDP) per

viable cell (MTT assay) indicates that the net enzymatic activity of MSCs from postmenopausal, but not from younger females, increases 3 to 5 fold as cultures progress from day 7 to 21. Given that the number of viable cells did not increase proportionally in the same time period, the results suggest that more differentiated MSCs exhibit higher NTPDase activity implicated in the extracellular inactivation of both UTP and UDP. The presence of NTPDase3 in postmenopausal MSCs and its absence in the cells from younger females may give a good explanation for the discrepancy between the two age groups concerning the ability to hydrolyse uracil nucleotides and their impact on intracellular $[Ca^{2+}]_i$ signalling and commitment to osteogenic differentiation. Interestingly, 7-day cultures of MSCs from postmenopausal women metabolise significantly ($P<0.05$) less UTP than that observed in cultures from younger patients (Figure 25Biii). This may be attributable to the relatively low expression levels of NTPDase2 (CD39LI, EC 3.6.1.3) in 7-day cultures of postmenopausal MSCs (see Figure 24B), as this enzyme is a preferential nucleoside triphosphatase hydrolysing diphosphates 10-15 less efficiently than triphosphates (Kukulsky et al., 2005). Taking into consideration that NTPDases prefer adenine nucleotides to uracil nucleotides as substrates, the presence of low amounts of NTPDase1 and -3 may not be enough to compensate UTP hydrolysis by absent NTPDase2 in 7-day postmenopasual MSCs.

Increases in the ATP content of the samples collected from postmenopausal MSCs grown for 21 days in culture clearly contrasts with the faster extracellular inactivation kinetics of the nucleotide upon cells maturation (7<21 days) (Figure 25A), which was also predicted by the concomitant gain of expression of NTPDases in more differentiated cells (Figure 24B). This apparent discrepancy can only be resolved assuming that differentiated MSCs from postmenopausal women release constitutively more ATP than cells from younger females by a yet unknown mechanism, excluding *a priori* cells damage considering the low levels of LDH activity measured in the same samples. Human MSCs and osteoblast-like cells release ATP constitutively without cell damage (see *e.g.* Brandao-Burch et al., 2012; Buckley et al., 2003; Genetos et al., 2005; Riddle et al., 2007;

Romanello et al., 2001). There are two main mechanisms underlying nonlytic release of nucleotides from cells: (a) Exocytic release specifically concentrated within secretory granules or vesicles; and (b) controlled release of cytosolic nucleotides via intrinsic plasma membranes channels or pores, which includes ABC transporters, connexin hemichannels voltage-dependent anion channels and the P2X7 receptor itself (Donahue, 2000; Orriss et al., 2009; Riddle et al., 2007; reviewed in Burnstock, 2006). Evidence now suggests that stimuli inducing bone formation and accelerated fracture healing (like mechanical loading, ultrasound, 1,25/OH)$_2$ vitamin D$_3$, and biphosphonates) promote ATP release from osteoblast-like cells (Alvarenga et al., 2010; Biswas and Zanello, 2009; Hayton et al., 2005; Riddle et al., 2007; Romanello et al., 2006; Rumney et al., 2012). Interestingly, the amount of ATP released from osteoblasts also depends on their differentiation status (Orriss et al., 2009). Using rat osteoblasts, these authors demonstrated that mature bone-forming cells release up to 7-fold more ATP than proliferative immature cells, yet we could not reproduce the same tendency in MSCs from young females. As a matter of fact, we found higher endogenous levels of ATP in the culture medium of more differentiated MSCs from postmenopausal women. Despite this, we were unable to demonstrate any tonic activity of the P2X7 receptor in postmenopausal MSCs using the selective receptor antagonist A438079 in a concentration (3 μM) that almost prevented ALP activity in cells from younger females (Noronha-Matos et al., 2014). This difference may be attributable (1) to a slight decline in the P2X7 receptor expression, and/or (2) to an increase in the breakdown of ATP by highly expressed NTPDases in the close proximity of P2X7 receptors expressed in more differentiated postmenopausal MSCs.

Like ATP, the ability of various cell types (*e.g.* murine airway epithelial cells, human cardiomyocytes) to release UTP has been directly confirmed using an enzymatic assay; estimated UTP release was approximately 10-30% of all detected nucleotides (Lazarowski and Harden, 1999; Lazarowski et al., 2003). In addition, UDP-sugars, like UDP-glucose (P2Y$_{14}$ receptor activator), may be released from cells together with ATP under certain conditions (Kreda et al., 2007, 2008; Lazarowski et al., 2003). UDP itself is an end product of glycogen synthesis

that may be released to the extracellular fluid. It is important to stress that the extracellular accumulation of nucleotides such as UTP and UDP may not necessarily involve a requirement for their selective release from intracellular pools. Rather these nucleotides may accumulate as a secondary consequence of extracellular metabolism (degradation or synthesis) of nucleotide precursors that are directly released in various physiological and pathological conditions (Burrell et al., 2005; Buxton et al., 2001; Joseph et al., 2004; Yegutkin et al., 2001; 2002). Thus, ADP or UDP may either be released directly or be generated via the extracellular hydrolysis of released ATP and UTP by NTPDases.

Interestingly, UTP is a much better substrate than UDP for NTPDase1 and NTPDase3 (Kukulski et al., 2005), which may influence the real potency of UTP on $P2Y_2$ and $P2Y_4$ receptors expressed on MSCs in culture. Distinctively from adenine nucleotides, all membrane-bound NTPDases dephosphorylate UTP with a transient formation of UDP, thus favouring $P2Y_6$ receptor activation (Kukulski et al., 2005; see Figure 23 and Table 5). Expression of NTPDase2 in more differentiated MSCs from postmenopausal women is expected to terminate ATP and UTP actions on P2X7 and $P2Y_{2,4}$ receptors, respectively. Simultaneously, increased NTPDase2 activity provide the generation of UDP for stimulation of $P2Y_6$ receptors, which may be balanced through the concomitant increase in the expression of NTPDase1 and -3, which efficiently hydrolyse UDP. These changes may determine the decay of ATP, UTP and UDP actions on intracellular $[Ca^{2+}]_i$ signals and ALP activity in more differentiated postmenopausal MSCs in culture, despite the presence of available P2 purinoceptors. At this stage, one could not completely rule out the influence of adenosine accumulation (or its lack) originated from the extracellular catabolism of adenine nucleotides *via* ecto-5'-nucleotidase/CD73, since the nucleoside is also an important regulator of osteogenic differentiation through the activation of subtype-specific P1 receptors, namely the most abundant A_{2B} receptor in human MSCs (Costa et al., 2011).

Comparing the expression and activity of NTPDases in young females and postmenopausal women it became clear that high NTPDase3 activity determines down-modulation of the activity of bone-promoting P2X7 and $P2Y_6$ receptors,

which could be readmitted by selectively inhibiting its activity with PSB 06126 or with a monoclonal antibody against the human enzyme, hN3-B3$_S$. Inhibition of NTPDase3 rescued osteogenic transcription factors (Runx-2 and Osterix), ALP activity, and matrix mineralization of postmenopausal MSCs to the levels observed in younger females, suggesting that age-dependent expression of this enzyme may be implicated in bone mass loss due to unbalance of the purinergic commitment of MSCs to osteogenic differentiation in postmenopausal women. The exact trigger and the timing associated with age-related expression of NTPDase3 in MSCs remains uncertain. Preliminary data from our laboratory, show that NTPDase3 was also absent from cultured MSCs isolated from a 39-years old man requiring bone engraftment due to traumatic bone fracture, as well as from a 50-years old male undergoing total hip replacement due to osteoarthrosis (data not shown). In these patients, the expression pattern of NTPDase1 and -2 resemble that found in the younger female group. It, thus, appears that oestrogen deficiency after menopause may be a trigger worth to investigate concerning bone loss linked to overexpression of NTPDase3 and P2 receptor activity impairment in postmenopausal women.

In conclusion, inhibition of NTPDase3 on MSCs may be a novel therapeutic strategy to increase bone formation in postmenopausal women, which together with activation of bone-forming P2X7 and P2Y$_6$ receptors may increase osteogenesis and matrix mineralization in situations where bone destruction exceeds bone formation (*e.g.* osteoporosis, rheumatoid arthritis, osteogenesis imperfect, fracture mal-union).

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4. DISCUSSION AND CONCLUSIONS

Bone is a dynamic tissue that is constantly being remodelled. This allows it to maintain its healthy condition, which is crucial for the efficient and lifelong execution of important skeletal functions (see Introduction, section 1.3). The development of novel therapies to overcome bone loss is of utmost importance. Some of the current therapeutic approaches (see Introduction, section 1.3.3) lack specificity and/or present countless side effects. Among bone diseases, osteoporosis has become a major public health problem in most developed countries. Only in USA, it affects at least 10 million citizens over the age of 50, and an additional 33–34 million Americans complain from osteopenia. Moreover, twenty-six percent of women over age 65 have osteoporosis (Feng and McDonald, 2011).

Bone tissue engineering (BTE) arose as a promising strategy to prevent bone loss, given the possibility of using osteoprogenitor cells supplemented with growth factors to promote new bone formation at defective sites. This presents an elegant approach, which includes minimal-invasive surgical approaches for the management of bone loss. However, some important issues must be taken into consideration. For obvious reasons, the use of the patient's own cells is most attractive in order to avoid the potential for immune rejection. Usually, osteoprogenitor cells (mesenchymal stem cells, MSCs) derive from the patient's own bone marrow. These are easily isolated and have a high proliferative capacity, while retaining their undifferentiated state (Banfi et al., 2002; Bruder et al., 1997). These cells, under the appropriate conditions, can differentiate into osteoblasts, the bone forming cells (Bobis et al., 2006). However, such ability becomes compromised in elderly individuals, which may result in bone disorders such as osteoporosis (see Introduction, section 1.2.3), thus limiting their usage in the context of bone tissue engineering (BTE) and cell transplantation procedures (Raggi and Berardi, 2012). Some of these alterations have been previously explored reinforcing the idea that ageing is partly due to a decline of stem cell function. As mentioned previously in this work (see section 1.2.3), possible

explanations for this fact may lie at the molecular level. Ageing is associated to an accumulation of cells damage affecting DNA, proteins, membranes and organelles (Ju and Rudoplh, 2008). Regarding bone cells, it was previously mentioned that a decline in oestrogen levels after menopause affects osteogenic differentiation; interestingly, oestrogen decline and telomere shortening (and so DNA damage) have been linked (Bayne et al., 2011). Another intriguing possibility that may explain stem cell loss of function is an alteration at local factors constitutively released by these cells. These molecules are required, together with multiple systemic factors, as important regulators of cell proliferation and/or differentiation fate. Purines and pyrimidines belong to such a group of local signalling molecules that are released to bone microenvironment under physiological conditions (*e.g*. mechanical loading, circulating hormones), but whose levels dramatically increase in response to bone injury (*e.g*. inflammation, hypoxia) (see sections 1.1.3 and 1.4).

In order to improve stem cell usage in BTE, it is crucial to understand the mechanisms underlying the decline of stem cells function and to explore the local mediators that may help to ameliorate this condition. Purinergic signalling has emerged as a promising field in the context of bone remodelling. Nucleotides (*e.g*. ATP, UTP) are important local regulators of osteogenic differentiation of MSCs (see section 1.4). These are constitutively released by cells under basal and/or stressful conditions and by binding to multiple P2 purinoceptors (P2Y and P2X) on osteoblasts and osteoclasts, may act as autocrine/paracrine regulators of bone remodelling (Burnstock et al., 2013). Purinergic signalling effects are influenced by multiple factors including the receptor subtype, the extracellular nucleotides present locally and the expression of ecto-nucleotidases which will, in turn, regulate nucleotide levels (see Burnstock et al., 2013). However, fine-tuning of such a complex set of interactions among the various players of the purinergic system is still highly controversial. The interplay between purinoceptor subtypes and ecto-enzymes on bone cells is still largely unknown leading to a gap in our knowledge regarding how they are organized to control bone remodelling. In addition, we must be aware that most of the highlights on this subject that are

available from the literature derive from using animal models and immortalized human cell lines, whilst only a few studies were carried out in non-modified human osteoprogenitors / osteoblasts.

For the reasons stated above, this work emerged to clarify some previously unknown molecular mechanisms operating upstream and downstream of P2 receptors in human osteogenesis, in both health and disease, in order to disclose new potential therapeutic targets. So, this work focused on three major goals: **(1)** to investigate the expression and function of uracil nucleotide-sensitive receptors ($P2Y_2$, $P2Y_4$, and $P2Y_6$) in human bone marrow MSCs; **(2)** to study the expression and function of P2X7 receptors on osteogenic differentiation of bone marrow MSCs in culture, exploring the underlying mechanism involved; **(3)** to disclose the importance of NTPDases in the management of cell differentiation and/or proliferation of human MSCs.

In this study, we demonstrated that isolated human bone marrow-derived MSCs express important stem cell markers, namely CD29, CD105, CD117 and CD73. Conversely, these cells were negative for haematopoietic surface markers, like CD14 and CD45 (Noronha-Matos et al., 2012). These findings suggest that our cultures were enriched in MSCs. MSCs allowed to grow in culture with an osteogenic-inducing medium synthetize type I collagen and osteocalcin, exhibit alkaline phosphatase (ALP) activity, express osteogenic transcription factors like Runx-2 and Osterix, and, finally, form bone-nodules of mineralized matrix at latter culture stages. This osteogenic differentiation capability is compromised in postmenopausal woman when compared to younger females, without being accompanied by changes in proliferation (Noronha-Matos et al., 2012).

Exploitation of the role of purinergic signalling between younger females and postmenopausal women revealed previously unknown information. Data show that the expression of P2 receptors is age and differentiation-dependent, as it was shown in rat osteoprogenitor cells (Orriss et al. 2006). In young females, immunoreactivity against P2X7 and $P2Y_6$ increases with time of the cells in culture ($7<21$ days); the opposite was observed regarding the expression of $P2Y_2$ and $P2Y_4$ receptors. In the postmenopausal group, the $P2Y_2$ and $P2Y_4$ receptors

immunoreactivity increase with time of the cells in culture (7<21 days), whereas the P2X7 receptor expression decrease slightly as cells differentiate and $P2Y_6$ receptor remains fairly constant throughout the culture period (7~21 days). Such differences necessarily implicate functional consequences in the context of purinergic signalling. Higher expression levels of osteogenic inducing receptors, such as $P2Y_6$ and P2X7 receptors, in MSCs from younger females predict enhanced osteogenic potential as compared with the cells from postmenopausal women (Noronha-Matos et al., 2012; 2014). In contrast, the latter express higher levels of $P2Y_2$ and $P2Y_4$ receptors upon maturation, which are known to inhibit bone formation when exposed to both ATP and UTP (Hoebertz et al., 2002), thus counteracting bone formation induced by UDP and ATP via co-expressed $P2Y_6$ and P2X7 receptors, respectively. On its own, this receptor pattern might explain the differences in the osteogenic potential found between cells from the two age groups. We observed in the postmenopausal group that the expression of the P2X7 receptor declines slightly throughout the culture period, which may additionally contribute to the osteogenic differentiation impairment of MSCs from postmenopausal women compared to younger females.

From data presented in this study, it became evident that UDP-sensitive $P2Y_6$ and ATP-activated P2X7 receptors are important promoters of osteogenesis and matrix mineralization. Unrestrained activation of $P2Y_6$ receptors with a stable UDP analogue (PSB 0474), but not with the non-hydrolysable UTP analogue (UTPγS) favoured intracellular $[Ca^{2+}]_i$ oscillations and increased ALP activity in MSC cultures. Nevertheless, the actions of native uracil nucleotides (UDP and UTP) declined significantly as MSC cultures progress to a less proliferative and more differentiated stage, because their effects may be balanced through nucleotide metabolizing enzymes (NTPDases), which determine whether osteoblast progenitors are driven into proliferation or differentiation (Noronha-Matos et al., 2012). Our data regarding the action of the P2X7 receptor on the osteogenic commitment of MSCs confirmed epidemiological studies suggesting that loss of function polymorphisms of the P2X7 receptor has been associated with increased risk of fractures in postmenopausal women. Results presented here demonstrate

that activation of the P2X7 receptor increase $[Ca^{2+}]_i$ accumulation in parallel to the formation of reversible plasma membrane blebs and pores permeable to high-molecular weight fluorescent dyes (*e.g.* TO-PRO-3). The way P2X7 receptors promote osteogenic differentiation (increase in ALP activity and in the expression of osteogenic transcription markers, Runx-2 and Osterix) and mineralization of postmenopausal MSCs in culture is independent of $[Ca^{2+}]_i$, but involves cytoskeleton rearrangements secondary to the activation of a PLC/PKC/Rho-kinase pathway. These findings contributed to clarify previously obtained results regarding the role of the P2X7 receptor in osteogenic differentiation of human osteoprogenitor cells, which have been extrapolated from data obtained in different species often with conflicting results (see *e.g*. Roger et al., 2010). Most importantly, our findings point towards new pharmacological targets (the P2X7 receptor and downstream signalling cascade) for the therapeutic management of bone disorders leading to increased bone loss, osteoporosis and fracture risk.

Activation of P2 purinoceptors may be balanced through the catabolism of nucleotides by extracellular enzymes, namely NTPDases. Our findings show, for the first time, that the actions of both adenine and uracil nucleotides on the osteogenic commitment of MSCs largely depend on the kinetics of their extracellular hydrolysis by NTPDases. Thus, beyond age and differentiation-dependent P2 receptors activity, a second degree of complexity emerges due to the differential expression of certain NTPDase subtypes between young females and postmenopausal women, which ultimately determines the amount and type of nucleotides reaching P2 receptors depending on the maturation stage of the cells. We show here, for the first time, that MSCs from young females do not express NTPDase3, but this enzyme is highly expressed in more differentiated cells from postmenopausal women (Figure 29). On the other hand, while young females express consistently both NTPDase1 and 2 along the differentiation process, these enzymes only gain significant expression in less proliferative and more differentiated cells from postmenopausal women. These differences, together with the reported changes with age and differentiation status of MSCs among young

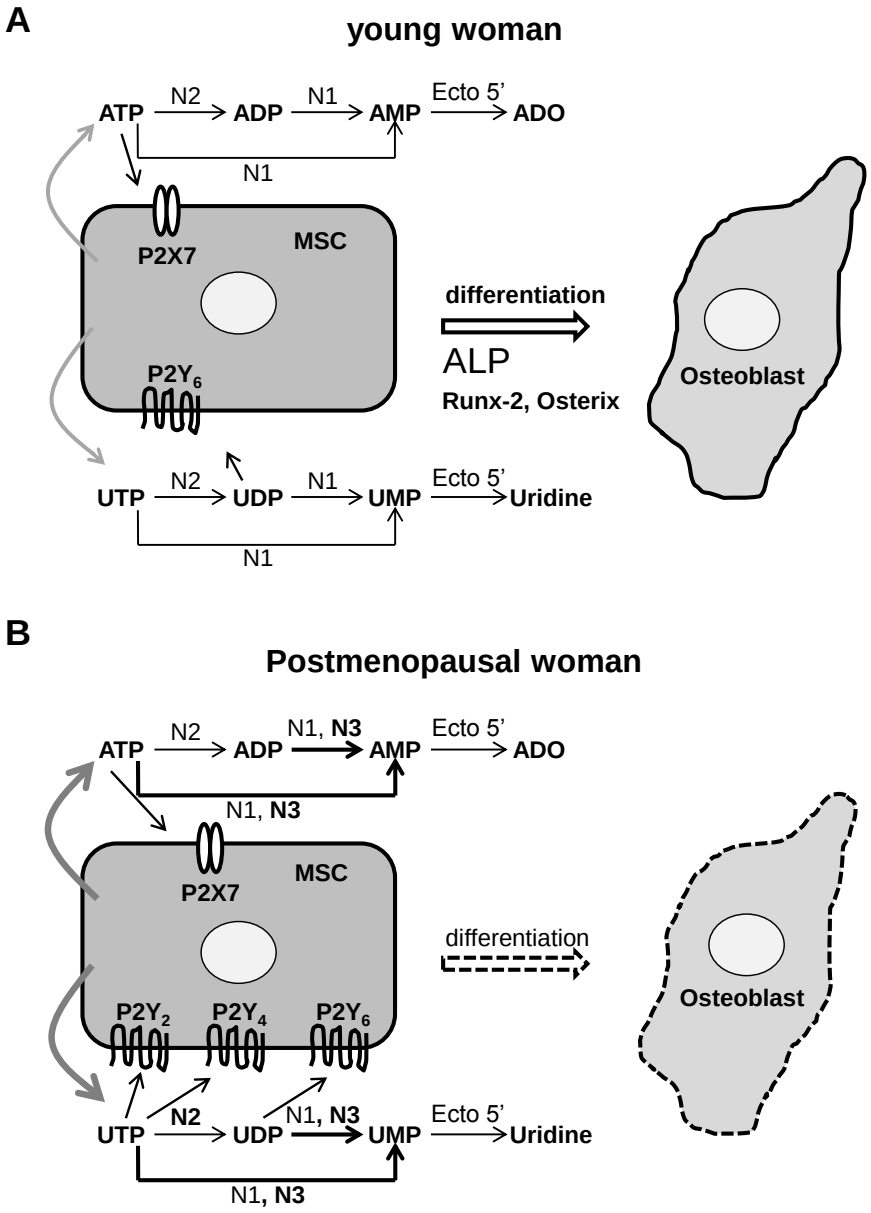

Figure 29. Osteogenic differentiation of bone marrow-derived mesenchymal stem cells (MSCs) is compromised in postmenopausal woman. **(A)** Representative diagram of a MSC under osteogenic differentiation from a young and healthy female. Nucleotides (like ATP and UTP) are constitutively released to the extracellular medium and converted into different metabolites throughout the NTPDase cascade, allowing the activation of different P2 receptors, namely P2X7 and $P2Y_6$ receptors. Such activation promotes $[Ca^{2+}]_i$ oscillations ($P2Y_6$ and P2X7 receptor) as well as reversible plasma membrane zeiosis (P2X7 receptor), increases the ALP activity (P2X7 and $P2Y_6$ receptors) and the expression of transcription factors like Runx-2 and Osterix (P2X7 receptor), which are involved in osteogenic differentiation of MSCs. Such mechanism(s) causes the commitment of the MSC to become a mature osteoblast, a cell capable of new bone formation (mineralization). **(B)** Representative diagram of a MSC under osteogenic differentiation from a postmenopausal woman.

Figure 29. *(Continued)* Nucleotides (such as ATP and UTP) are constitutively released in higher amounts as compared to MSCs from younger individuals (diagram A). Nucleotide inactivation (or catabolism) via NTPDases is faster as compared to younger females, since these cells over express NTPDase3. This compromises ATP and UDP endogenous levels, and so P2X7 and $P2Y_6$ receptors activation, respectively, leading to impairment of osteogenic differentiation. In addition, these cells express $P2Y_2$ and $P2Y_4$ receptors which have been associated with the inhibition of cell differentiation. This will compromise the formation of new bone (mineralization). Grey arrows represent ATP or UTP release (bold arrow means higher nucleotide release); black arrows represent nucleotide breakdown (bold arrows means higher catabolism rate), P2 receptor's activation, or cell differentiation; dashed arrows or lines means compromised cell differentiation. Mesenchymal stem cell, MSC; NTPDase1, N1; NTPDase2, N2; NTPDase3, N3; ecto 5'-nucleotidase/CD73, Ecto 5'.

females and postmenopausal women, may contribute to explain (at least in part) the divergence in the osteogenic potential between the two age groups shedding light to find novel putative targets to increase bone formation (Figure 29). The most striking difference regarding the purinergic signalling pathway found between young females and postmenopausal women was that the lack of NTPDase3 in the younger group; the same was recently confirmed in men. This enzyme, as mentioned, compromises the activity of the osteogenic inducers P2X7 and $P2Y_6$ receptors (Figure 29). This difference may give a good explanation for the discrepancy between the two age groups concerning the ability to hydrolyse uracil nucleotides and their impact on intracellular $[Ca^{2+}]_i$ signalling and commitment to osteogenic differentiation. In contrast to adenine nucleotides, all membrane-bound NTPDases dephosphorylate UTP with a transient formation of UDP, thus favouring $P2Y_6$ receptor activation (Kukulski et al., 2005). In younger females, formation of nucleotide diphosphates (namely UDP) by NTPDase2 is unrestrained by the absence of NTPDase3, a situation that does not occur in postmenopausal women.

The deleterious influence of overexpression of NTPDase3 on osteogenic commitment of postmenopausal MSCs (Figure 29) was confirmed in experiments using the selective inhibitor of this enzyme, PSB 06126, or an antibody directed against the human enzyme, $hN3\text{-}B3_s$. Selective inhibition of NTPDase3 in postmenopausal MSCs increased the amount of ATP in the culture medium and rescued the osteogenic phenotype observed in younger females concerning increases in ALP activity, in the levels of osteogenic factors (Runx-2 and Osterix) and in the ability to form mineralized bone-nodules. Using NTPDase3 inhibitors we

also readmitted tonic activation of P2X7 and $P2Y_6$ receptors by endogenously released adenine and uracil nucleotides, which had been lost in postmenopausal women as determined by the absence of the effect of specific receptor antagonists, respectively A438079 and MRS 2578, in concentrations that significantly decreased osteogenic differentiation in younger females (Noronha-Matos et al., 2012; 2014).

All together, our results suggest that there are three major potential therapeutic targets on the purinergic cascade which may be used for the management of bone loss: the UDP-sensitive $P2Y_6$ receptor (Noronha-Matos et al., 2012), the ATP-sensitive P2X7 receptor (Noronha-Matos et al., 2014), and the ecto-NTPDase3 enzyme (manuscript in preparation). We strongly believe that these results will provide new insights for development of novel therapeutic strategies to increase osteogenesis and matrix mineralization in situations where bone destruction exceeds bone formation (*e.g*. osteoporosis, rheumatoid arthritis, osteogenesis imperfect, fracture mal-union). In addition, our findings provide the basis to promote osteogenic differentiation of postmenopausal MSCs *in vitro* in the context of BTE or cell therapy/transplantation procedures.

5. REFERENCES

Aaseth, J., Boivin, G., and Andersen, O. 2012. Osteoporosis and trace elements-an overview. *J Trace Elem Med Biol*. 26:149-152.

Abbracchio, M.P., and Burnstock, G. 1994. Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther*. 64:445-475.

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. 2006. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature*. 439:599-603.

Adinolfi, E., Cirillo, M., Woltersdorf, R., Falzoni, S., Chiozzi, P., Pellegatti, P., Callegari, M.G., Sandona, D., Markwardt, F., Schmalzing, G., and Di Virgilio, F. 2010. Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. *FASEB J*. 24:3393-3404.

Adler, C.P. 2000. Bones and bone tissue: normal anatomy and histology. *In* Bone Diseases. *Springer-Verlag. 1st edition. New York*: 1-30

Aiuti, A., Tavian, M., Cipponi, A., Ficara, F., Zappone, E., Hoxie, J., Peault, B., and Bordignon, C. 1999. Expression of CXCR4, the receptor for stromal cell-derived factor-1 on fetal and adult human lympho-hematopoietic progenitors. *Eur J Immunol*. 29:1823-1831.

Alloisio, S., Di Garbo, A., Barbieri, R., Bozzo, L., Ferroni, S., and Nobile, M. 2010. Evidence for two conductive pathways in P2X receptor: differences in modulation and selectivity. *J Neurochem*. 113:796-806.

Allsopp, R.C., Cheshier, S., and Weissman, I.L. 2001. Telomere shortening accompanies increased cell cycle activity during serial transplantation of hematopoietic stem cells. *J Exp Med*. 193:917-924.

Allsopp, R.C., Morin, G.B., DePinho, R., Harley, C.B., and Weissman, I.L. 2003. Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. *Blood*. 102:517-520.

Almeida, M., Han, L., Martin-Millan, M., Plotkin, L.I., Stewart, S.A., Roberson, P.K., Kousteni, S., O'Brien, C.A., Bellido, T., Parfitt, A.M., Weinstein, R.S., Jilka, R.L.,

and Manolagas, S.C. 2007. Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. *J Biol Chem*. 282:27285-27297.

Alqallaf, S.M., Evans, B.A., and Kidd, E.J. 2009. Atypical P2X receptor pharmacology in two human osteoblast-like cell lines. *Br J Pharmacol*. 156:1124-1135.

Alvarenga, E.C., Rodrigues, R., Caricati-Neto, A., Silva-Filho, F.C., Paredes-Gamero, E.J., and Ferreira, A.T. 2010. Low-intensity pulsed ultrasound-dependent osteoblast proliferation occurs by via activation of the P2Y receptor: role of the P2Y1 receptor. *Bone*. 46:355-362.

Amaral, M., Costa, M.A., Lopes, M.A., Silva, R.F., Santos, J.D., and Fernandes, M.H. 2002. Si(3)N(4)-bioglass composites stimulate the proliferation of MG63 osteoblast-like cells and support the osteogenic differentiation of human bone marrow cells. *Biomaterials*. 23:4897-4906.

Anderson, P.H., Lam, N.N., Turner, A.G., Davey, R.A., Kogawa, M., Atkins, G.J., and Morris, H.A. 2013. The pleiotropic effects of vitamin D in bone. *J Steroid Biochem Mol Biol*. 136:190-194.

Ankrom, M.A., Patterson, J.A., d'Avis, P.Y., Vetter, U.K., Blackman, M.R., Sponseller, P.D., Tayback, M., Robey, P.G., Shapiro, J.R., and Fedarko, N.S. 1998. Age-related changes in human oestrogen receptor alpha function and levels in osteoblasts. *Biochem J*. 333:787-794.

Antoniazzi, F., Mottes, M., Fraschini, P., Brunelli, P.C., and Tato, L. 2000. Osteogenesis imperfecta: practical treatment guidelines. *Paediatr Drugs*. 2:465-488.

Aref, M., Gallant, M.A., Organ, J.M., Wallace, J.M., Newman, C.L., Burr, D.B., Brown, D.M., and Allen, M.R. 2013. In vivo reference point indentation reveals positive effects of raloxifene on mechanical properties following 6 months of treatment in skeletally mature beagle dogs. *Bone*. 56:449-453.

Armstrong, S., Pereverzev, A., Dixon, S.J., and Sims, S.M. 2009. Activation of P2X7 receptors causes isoform-specific translocation of protein kinase C in osteoclasts. *J Cell Sci*. 122:136-144.

Arnsdorf, E.J., Tummala, P., Kwon, R.Y., and Jacobs, C.R. 2009. Mechanically induced osteogenic differentiation--the role of RhoA, ROCKII and cytoskeletal dynamics. *J Cell Sci*. 122:546-553.

Atkins, G.J., Anderson, P.H., Findlay, D.M., Welldon, K.J., Vincent, C., Zannettino, A.C., O'Loughlin, P.D., and Morris, H.A. 2007. Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D3. *Bone*. 40:1517-1528.

Atkins, G.J., Welldon, K.J., Halbout, P., and Findlay, D.M. 2009. Strontium ranelate treatment of human primary osteoblasts promotes an osteocyte-like phenotype while eliciting an osteoprotegerin response. *Osteoporos Int*. 20:653-664.

Augello, A., and De Bari, C. 2010. The regulation of differentiation in mesenchymal stem cells. *Hum Gene Ther*. 21:1226-1238.

Augello, A., Kurth, T.B., and De Bari, C. 2010. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater*. 20:121-133.

Avalos, A.M., Valdivia, A.D., Munoz, N., Herrera-Molina, R., Tapia, J.C., Lavandero, S., Chiong, M., Burridge, K., Schneider, P., Quest, A.F., and Leyton, L. 2009. Neuronal Thy-1 induces astrocyte adhesion by engaging syndecan-4 in a cooperative interaction with alphavbeta3 integrin that activates PKCalpha and RhoA. *J Cell Sci*. 122:3462-3471.

Avery, S., Inniss, K., and Moore, H. 2006. The regulation of self-renewal in human embryonic stem cells. *Stem Cells Dev*. 15:729-740.

Baddoo, M., Hill, K., Wilkinson, R., Gaupp, D., Hughes, C., Kopen, G.C., and Phinney, D.G. 2003. Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem*. 89:1235-1249.

Baker, J.C., and Harland, R.M. 1997. From receptor to nucleus: the Smad pathway. *Curr Opin Genet Dev*. 7:467-473.

Banfi, A., Bianchi, G., Notaro, R., Luzzatto, L., Cancedda, R., and Quarto, R. 2002. Replicative aging and gene expression in long-term cultures of human bone marrow stromal cells. *Tissue Eng*. 8:901-910.

Baqi, Y., Weyler, S., Iqbal, J., Zimmermann, H., and Muller, C.E. 2009. Structure-activity relationships of anthraquinone derivatives derived from bromaminic acid as inhibitors of ectonucleoside triphosphate diphosphohydrolases (E-NTPDases). *Purinergic Signal*. 5:91-106.

Baran, D.T., and Braverman, L.E. 1991. Thyroid hormones and bone mass. *J Clin Endocrinol Metab*. 72:1182-1183.

Baron, R. 1993. Anatomy and Ultrastructure of Bone*. In* Primer *on the* Metabolic Bone Diseases and Disorders of Mineral Metabolism. *2nd edition. Favus M., J. Raven Press. New York.* 1:3-9.

Baylink, D.J. 1983. Glucocorticoid-induced osteoporosis. *N Engl J Med*. 309:306-308.

Bayne, S., Li, H., Jones, M.E., Pinto, A.R., van Sinderen, M., Drummond, A., Simpson, E.R., and Liu, J.P. 2011. Estrogen deficiency reversibly induces telomere shortening in mouse granulosa cells and ovarian aging in vivo. *Protein Cell*. 2:333-346.

Beauregard, M., Gagnon, E., Guay-Belanger, S., Siris, E.S., Morissette, J., Brown, J.P., and Michou, L. 2013. Genetic association study of Dickkopf-1 and sclerostin genes with paget disease of bone. *Calcif Tissue Int*. 93:405-412.

Beck, G.R., Jr., Zerler, B., and Moran, E. 2000. Phosphate is a specific signal for induction of osteopontin gene expression. *Proc Natl Acad Sci U S A*. 97:8352-8357.

Behr, B., Ko, S.H., Wong, V.W., Gurtner, G.C., and Longaker, M.T. 2010. Stem cells. *Plast Reconstr Surg*. 126:1163-1171.

Bellido, T., Jilka, R.L., Boyce, B.F., Girasole, G., Broxmeyer, H., Dalrymple, S.A., Murray, R., and Manolagas, S.C. 1995. Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor. *J Clin Invest*. 95:2886-2895.

Bellows, C.G., Aubin, J.E., and Heersche, J.N. 1991. Initiation and progression of mineralization of bone nodules formed in vitro: the role of alkaline phosphatase and organic phosphate. *Bone Miner*. 14:27-40.

Benayahu, D., Kletter, Y., Zipori, D., and Wientroub, S. 1989. Bone marrow-derived stromal cell line expressing osteoblastic phenotype in vitro and osteogenic capacity in vivo. *J Cell Physiol*. 140:1-7.

Bianco, P., Silvestrini, G., Ballanti, P., and Bonucci, E. 1992. Paramyxovirus-like nuclear inclusions identical to those of Paget's disease of bone detected in giant cells of primary oxalosis. *Virchows Arch A Pathol Anat Histopathol*. 421:427-433.

Bigonnesse, F., Levesque, S.A., Kukulski, F., Lecka, J., Robson, S.C., Fernandes, M.J., and Sévigny, J. 2004. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. *Biochemistry*. 43:5511-5519.

Bilezikian, J.P. 2002. Sex steroids, mice, and men: when androgens and estrogens get very close to each other. *J Bone Miner Res*. 17:563-566.

Bisello, A., Horwitz, M.J., and Stewart, A.F. 2004. Parathyroid hormone-related protein: an essential physiological regulator of adult bone mass. *Endocrinology*. 145:3551-3553.

Biswas, P., and Zanello, L.P. 2009. 1alpha,25(OH)(2) vitamin D(3) induction of ATP secretion in osteoblasts. *J Bone Miner Res*. 24:1450-1460.

Biver, G., Wang, N., Gartland, A., Orriss, I., Arnett, T.R., Boeynaems, J.M., and Robaye, B. 2013. Role of the P2Y13 receptor in the differentiation of bone marrow stromal cells into osteoblasts and adipocytes. *Stem Cells*. 31:2747-2758.

Blackburn, E.H. 1991. Structure and function of telomeres. *Nature*. 350:569-573.

Blackburn, E.H. 2001. Switching and signalling at the telomere. *Cell*. 106:661-673.

Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J., and Bunting, S. 1990. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J Pharmacol Exp Ther*. 255:756-768.

Bobis, S., Jarocha, D., and Majka, M. 2006. Mesenchymal stem cells: characteristics and clinical applications. *Folia Histochem Cytobiol*. 44:215-230.

Bodin, P., and Burnstock, G. 2001. Purinergic signalling: ATP release. *Neurochem Res*. 26:959-969.

Body, J.J. 2002. Calcitonin for the long-term prevention and treatment of postmenopausal osteoporosis. *Bone*. 30:75S-79S.

Boiret, N., Rapatel, C., Veyrat-Masson, R., Guillouard, L., Guerin, J.J., Pigeon, P., Descamps, S., Boisgard, S., and Berger, M.G. 2005. Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow. *Exp Hematol*. 33:219-225.

Bollerslev, J., Ueland, T., Landaas, S., and Marks, S.C., Jr. 2000. Serum creatine kinase isoenzyme BB in mammalian osteopetrosis. *Clin Orthop Relat Res*. 377:241-247.

Borgen, E., Beiske, K., Trachsel, S., Nesland, J.M., Kvalheim, G., Herstad, T.K., Schlichting, E., Qvist, H., and Naume, B. 1998. Immunocytochemical detection of isolated epithelial cells in bone marrow: non-specific staining and contribution by plasma cells directly reactive to alkaline phosphatase. *J Pathol*. 185:427-434.

Boudreaux, J.M., and Towler, D.A. 1996. Synergistic induction of osteocalcin gene expression: identification of a bipartite element conferring fibroblast growth factor 2 and cyclic AMP responsiveness in the rat osteocalcin promoter. *J Biol Chem*. 271:7508-7515.

Bowler, W.B., Birch, M.A., Gallagher, J.A., and Bilbe, G. 1995. Identification and cloning of human P2U purinoceptor present in osteoclastoma, bone, and osteoblasts. *J Bone Miner Res*. 10:1137-1145.

Bowler, W.B., Buckley, K.A., Gartland, A., Hipskind, R.A., Bilbe, G., and Gallagher, J.A. 2001. Extracellular nucleotide signalling: a mechanism for integrating local and systemic responses in the activation of bone remodelling. *Bone*. 28:507-512.

Bowler, W.B., Dixon, C.J., Halleux, C., Maier, R., Bilbe, G., Fraser, W.D., Gallagher, J.A., and Hipskind, R.A. 1999. Signalling in human osteoblasts by extracellular nucleotides. Their weak induction of the c-fos proto-oncogene via Ca2+ mobilization is strongly potentiated by a parathyroid hormone/cAMP-dependent protein kinase pathway independently of mitogen-activated protein kinase. *J Biol Chem*. 274:14315-14324.

Bowler, W.B., Littlewood-Evans, A., Bilbe, G., Gallagher, J.A., and Dixon, C.J. 1998. P2Y2 receptors are expressed by human osteoclasts of giant cell tumor but do not mediate ATP-induced bone resorption. *Bone*. 22:195-200.

Boyce, B.F. 2013. Advances in the regulation of osteoclasts and osteoclast functions. *J Dent Res*. 92:860-867.

Boyce, B.F., Rosenberg, E., de Papp, A.E., and Duong le, T. 2012. The osteoclast, bone remodelling and treatment of metabolic bone disease. *Eur J Clin Invest*. 42:1332-1341.

Boyce, B.F., and Xing, L. 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys*. 473:139-146.

Brandao-Burch, A., Key, M.L., Patel, J.J., Arnett, T.R., and Orriss, I.R. 2012. The P2X7 Receptor is an Important Regulator of Extracellular ATP Levels. *Front Endocrinol (Lausanne)*. 3:41.

Brandao-Burch, A., Utting, J.C., Orriss, I.R., and Arnett, T.R. 2005. Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. *Calcif Tissue Int*. 77:167-174.

Brekken, R.A., and Sage, E.H. 2001. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. *Matrix Biol*. 19:816-827.

Bruder, S.P., Jaiswal, N., and Haynesworth, S.E. 1997. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem*. 64:278-294.

Buckley, K.A., Golding, S.L., Rice, J.M., Dillon, J.P., and Gallagher, J.A. 2003. Release and interconversion of P2 receptor agonists by human osteoblast-like cells. *FASEB J*. 17:1401-1410.

Buckley, K.A., Wagstaff, S.C., McKay, G., Gaw, A., Hipskind, R.A., Bilbe, G., Gallagher, J.A., and Bowler, W.B. 2001. Parathyroid hormone potentiates nucleotide-induced [Ca2+]i release in rat osteoblasts independently of Gq activation or cyclic monophosphate accumulation. A mechanism for localizing systemic responses in bone. *J Biol Chem*. 276:9565-9571.

Burnstock, G. 2002. Purinergic signalling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol*. 22:364-373.

Burnstock, G. 2006. Purinergic signalling. *Br J Pharmacol*. 147:S172-181.

Burnstock, G. 2007. Purine and pyrimidine receptors. *Cell Mol Life Sci*. 64:1471-1483.

Burnstock, G., Arnett, T.R., and Orriss, I.R. 2013. Purinergic signalling in the musculoskeletal system. *Purinergic Signal*. 9:541-572.

Burnstock, G., and Kennedy, C. 1985. Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol*. 16:433-440.

Burnstock, G., and Knight, G.E. 2004. Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol*. 240:31-304.

Burnstock, G., and Verkhratsky, A. 2010. Long-term (trophic) purinergic signalling: purinoceptors control cell proliferation, differentiation and death. *Cell Death Dis*. 1:e9.

Burrell, H.E., Wlodarski, B., Foster, B.J., Buckley, K.A., Sharpe, G.R., Quayle, J.M., Simpson, A.W., and Gallagher, J.A. 2005. Human keratinocytes release ATP and utilize three mechanisms for nucleotide interconversion at the cell surface. *J Biol Chem*. 280:29667-29676.

Buxton, I.L., Kaiser, R.A., Oxhorn, B.C., and Cheek, D.J. 2001. Evidence supporting the Nucleotide Axis Hypothesis: ATP release and metabolism by coronary endothelium. *Am J Physiol Heart Circ Physiol*. 281:H1657-1666.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M., and Scadden, D.T. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 425:841-846.

Canalis, E. 1993. Systemic and local factors and the maintenance of bone quality. *Calcif Tissue Int*. 53:S90-92.

Cao, J.J., Kurimoto, P., Boudignon, B., Rosen, C., Lima, F., and Halloran, B.P. 2007. Aging impairs IGF-I receptor activation and induces skeletal resistance to IGF-I. *J Bone Miner Res*. 22:1271-1279.

Caplan, A.I., Elyaderani, M., Mochizuki, Y., Wakitani, S., and Goldberg, V.M. 1997. Principles of cartilage repair and regeneration. *Clin Orthop Relat Res*. 342:254-269.

Carrasquero, L.M., Delicado, E.G., Bustillo, D., Gutierrez-Martin, Y., Artalejo, A.R., and Miras-Portugal, M.T. 2009. P2X7 and P2Y13 purinergic receptors mediate intracellular calcium responses to BzATP in rat cerebellar astrocytes. *J Neurochem*. 110:879-889.

Carroll, S.H., Wigner, N.A., Kulkarni, N., Johnston-Cox, H., Gerstenfeld, L.C., and Ravid, K. 2012. A2B adenosine receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation in vivo. *J Biol Chem*. 287:15718-15727.

Carvalho, R.S., Schaffer, J.L., and Gerstenfeld, L.C. 1998. Osteoblasts induce osteopontin expression in response to attachment on fibronectin: demonstration of a common role for integrin receptors in the signal transduction processes of cell attachment and mechanical stimulation. *J Cell Biochem*. 70:376-390.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem*. 257:7847-7851.

Cheewatrakoolpong, B., Gilchrest, H., Anthes, J.C., and Greenfeder, S. 2005. Identification and characterization of splice variants of the human P2X7 ATP channel. *Biochem Biophys Res Commun*. 332:17-27.

Chen, J.R., Plotkin, L.I., Aguirre, J.I., Han, L., Jilka, R.L., Kousteni, S., Bellido, T., and Manolagas, S.C. 2005. Transient versus sustained phosphorylation and nuclear accumulation of ERKs underlie anti-versus pro-apoptotic effects of estrogens. *J Biol Chem*. 280:4632-4638.

Chen, Y., Shyu, J.F., Santhanagopal, A., Inoue, D., David, J.P., Dixon, S.J., Horne, W.C., and Baron, R. 1998. The calcitonin receptor stimulates Shc tyrosine phosphorylation and Erk1/2 activation. Involvement of Gi, protein kinase C, and calcium. *J Biol Chem*. 273:19809-19816.

Cheng, S.L., Yang, J.W., Rifas, L., Zhang, S.F., and Avioli, L.V. 1994. Differentiation of human bone marrow osteogenic stromal cells in vitro:

induction of the osteoblast phenotype by dexamethasone. *Endocrinology*. 134:277-286.

Chernausek, S.D., Backeljauw, P.F., Frane, J., Kuntze, J., and Underwood, L.E. 2007. Long-term treatment with recombinant insulin-like growth factor (IGF)-I in children with severe IGF-I deficiency due to growth hormone insensitivity. *J Clin Endocrinol Metab*. 92:902-910.

Chiu, C.P., Dragowska, W., Kim, N.W., Vaziri, H., Yui, J., Thomas, T.E., Harley, C.B., and Lansdorp, P.M. 1996. Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells*. 14:239-248.

Chow, J., Tobias, J.H., Colston, K.W., and Chambers, T.J. 1992. Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. *J Clin Invest*. 89:74-78.

Chung, C.Y., Iida-Klein, A., Wyatt, L.E., Rudkin, G.H., Ishida, K., Yamaguchi, D.T., and Miller, T.A. 1999. Serial passage of MC3T3-E1 cells alters osteoblastic function and responsiveness to transforming growth factor-beta1 and bone morphogenetic protein-2. *Biochem Biophys Res Commun*. 265:246-251.

Ciciarello, M., Zini, R., Rossi, L., Salvestrini, V., Ferrari, D., Manfredini, R., and Lemoli, R.M. 2013. Extracellular purines promote the differentiation of human bone marrow-derived mesenchymal stem cells to the osteogenic and adipogenic lineages. *Stem Cells Dev*. 22:1097-1111.

Clarke, B. 2008. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol*. 3:S131-139.

Coelho, M.J., Cabral, A.T., and Fernande, M.H. 2000. Human bone cell cultures in biocompatibility testing. Part I: osteoblastic differentiation of serially passaged human bone marrow cells cultured in alpha-MEM and in DMEM. *Biomaterials*. 21:1087-1094.

Coffin, J.D., Florkiewicz, R.Z., Neumann, J., Mort-Hopkins, T., Dorn, G.W., 2nd, Lightfoot, P., German, R., Howles, P.N., Kier, A., O'Toole, B.A., Sasse, J., Gonzalez, A.M., Baird, A., and Doetschman, T. 1995. Abnormal bone growth

and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Mol Biol Cell*. 6:1861-1873.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol*. 3:339-345.

Colgan, S.P., Eltzschig, H.K., Eckle, T., and Thompson, L.F. 2006. Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal*. 2:351-360.

Communi, D., Parmentier, M., and Boeynaems, J.M. 1996. Cloning, functional expression and tissue distribution of the human P2Y6 receptor. *Biochem Biophys Res Commun*. 222:303-308.

Conget, P.A., and Minguell, J.J. 1999. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol*. 181:67-73.

Costa, M.A., Barbosa, A., Neto, E., Sa-e-Sousa, A., Freitas, R., Neves, J.M., Magalhães-Cardoso, T., Ferreirinha, F., and Correia-de-Sá, P. 2011. On the role of subtype selective adenosine receptor agonists during proliferation and osteogenic differentiation of human primary bone marrow stromal cells. *J Cell Physiol*. 226:1353-1366.

Costessi, A., Pines, A., D'Andrea, P., Romanello, M., Damante, G., Cesaratto, L., Quadrifoglio, F., Moro, L., and Tell, G. 2005. Extracellular nucleotides activate Runx2 in the osteoblast-like HOBIT cell line: a possible molecular link between mechanical stress and osteoblasts' response. *Bone*. 36:418-432.

Couse, J.F., and Korach, K.S. 1999. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev*. 20:358-417.

Dalle Carbonare, L., Valenti, M.T., Zanatta, M., Donatelli, L., and Lo Cascio, V. 2009. Circulating mesenchymal stem cells with abnormal osteogenic differentiation in patients with osteoporosis. *Arthritis Rheum*. 60:3356-3365.

Danen, E.H., Lafrenie, R.M., Miyamoto, S., and Yamada, K.M. 1998. Integrin signalling: cytoskeletal complexes, MAP kinase activation, and regulation of gene expression. *Cell Adhes Commun*. 6:217-224.

Datta, H.K., Ng, W.F., Walker, J.A., Tuck, S.P., and Varanasi, S.S. 2008. The cell biology of bone metabolism. *J Clin Pathol*. 61:577-587.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 351:95-105.

D'Avis P, Y., Frazier, C.R., Shapiro, J.R., and Fedarko, N.S. 1997. Age-related changes in effects of insulin-like growth factor I on human osteoblast-like cells. *Biochem J*. 324:753-760.

De Bari, C., Dell'Accio, F., Vandenabeele, F., Vermeesch, J.R., Raymackers, J.M., and Luyten, F.P. 2003. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *J Cell Biol*. 160:909-918.

Deal, C. 2009. Potential new drug targets for osteoporosis. *Nat Clin Pract Rheumatol*. 5:20-27.

Deguchi, K., Yagi, H., Inada, M., Yoshizaki, K., Kishimoto, T., and Komori, T. 1999. Excessive extramedullary hematopoiesis in Cbfa1-deficient mice with a congenital lack of bone marrow. *Biochem Biophys Res Commun*. 255:352-359.

DeLucia, M.C., Mitnick, M.E., and Carpenter, T.O. 2003. Nutritional rickets with normal circulating 25-hydroxyvitamin D: a call for reexamining the role of dietary calcium intake in North American infants. *J Clin Endocrinol Metab*. 88:3539-3545.

Deng, Z.L., Sharff, K.A., Tang, N., Song, W.X., Luo, J., Luo, X., Chen, J., Bennett, E., Reid, R., Manning, D., Xue, A., Montag, A.G., Luu, H.H., Haydon, R.C., and He, T.C. 2008. Regulation of osteogenic differentiation during skeletal development. *Front Biosci*. 13:2001-2021.

Denhardt, D.T., and Guo, X. 1993. Osteopontin: a protein with diverse functions. *FASEB J*. 7:1475-1482.

Dennis, J.E., Carbillet, J.P., Caplan, A.I., and Charbord, P. 2002. The STRO-1+ marrow cell population is multipotential. *Cells Tissues Organs*. 170:73-82.

Devine, S.M., and Hoffman, R. 2000. Role of mesenchymal stem cells in hematopoietic stem cell transplantation. *Curr Opin Hematol*. 7:358-363.

Di Virgilio, F. 1995. The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. *Immunol Today*. 16:524-528.

Dixon, C.J., Bowler, W.B., Walsh, C.A., and Gallagher, J.A. 1997. Effects of extracellular nucleotides on single cells and populations of human osteoblasts: contribution of cell heterogeneity to relative potencies. *Br J Pharmacol*. 120:777-780.

Dixon, S.J., Kulaga, A., Jaworski, E.M., and Wilson, J.X. 1991. Ascorbate uptake by ROS 17/2.8 osteoblast-like cells: substrate specificity and sensitivity to transport inhibitors. *J Bone Miner Res*. 6:623-629.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 8:315-317.

Donahue, H.J. 2000. Gap junctions and biophysical regulation of bone cell differentiation. *Bone*. 26:417-422.

Donaldson, S.H., Lazarowski, E.R., Picher, M., Knowles, M.R., Stutts, M.J., and Boucher, R.C. 2000. Basal nucleotide levels, release, and metabolism in normal and cystic fibrosis airways. *Mol Med*. 6:969-982.

Dranoff, J.A., Kruglov, E.A., Toure, J., Braun, N., Zimmermann, H., Jain, D., Knowles, A.F., and Sevigny, J. 2004. Ectonucleotidase NTPDase2 is selectively down-regulated in biliary cirrhosis. *J Investig Med*. 52:475-482.

Driessen, G.J., Gerritsen, E.J., Fischer, A., Fasth, A., Hop, W.C., Veys, P., Porta, F., Cant, A., Steward, C.G., Vossen, J.M., Uckan, D., and Friedrich, W. 2003. Long-term outcome of haematopoietic stem cell transplantation in autosomal recessive osteopetrosis: an EBMT report. *Bone Marrow Transplant*. 32:657-663.

Dubyak, G.R. 2007. Go it alone no more--P2X7 joins the society of heteromeric ATP-gated receptor channels. *Mol Pharmacol*. 72:1402-1405.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L., and Karsenty, G. 1997. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*. 89:747-754.

Elder, C.J., and Bishop, N.J. 2014. Rickets. *Lancet*. 383:1665-1676.

El-Tayeb, A., Qi, A., and Muller, C.E. 2006. Synthesis and structure-activity relationships of uracil nucleotide derivatives and analogues as agonists at human P2Y2, P2Y4, and P2Y6 receptors. *J Med Chem*. 49:7076-7087.

Ernst, M., Heath, J.K., and Rodan, G.A. 1989. Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-I, and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. *Endocrinology*. 125:825-833.

Erson, A.E., and Petty, E.M. 2008. MicroRNAs in development and disease. *Clin Genet*. 74:296-306.

Ettinger, B., Black, D.M., Mitlak, B.H., Knickerbocker, R.K., Nickelsen, T., Genant, H.K., Christiansen, C., Delmas, P.D., Zanchetta, J.R., Stakkestad, J., Gluer, C.C., Krueger, K., Cohen, F.J., Eckert, S., Ensrud, K.E., Avioli, L.V., Lips, P., and Cummings, S.R. 1999. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *JAMA*. 282:637-645.

Evans, B.A., Elford, C., Pexa, A., Francis, K., Hughes, A.C., Deussen, A., and Ham, J. 2006. Human osteoblast precursors produce extracellular adenosine, which modulates their secretion of IL-6 and osteoprotegerin. *J Bone Miner Res*. 21:228-236.

Everts, V., Delaisse, J.M., Korper, W., Jansen, D.C., Tigchelaar-Gutter, W., Saftig, P., and Beertsen, W. 2002. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. *J Bone Miner Res*. 17:77-90.

Faria, R.X., Cascabulho, C.M., Reis, R.A., and Alves, L.A. 2010. Large-conductance channel formation mediated by P2X7 receptor activation is regulated through distinct intracellular signalling pathways in peritoneal macrophages and 2BH4 cells. *Naunyn Schmiedebergs Arch Pharmacol*. 382:73-87.

Feng, X., and McDonald, J.M. 2011. Disorders of bone remodeling. *Annu Rev Pathol*. 6:121-145.

Fernandes, M.H., Costa, M.A., and Carvalho, G.S. 1997. Mineralization in serially passaged human alveolar bone cells. *J Mater Sci Mater Med*. 8:61-65.

Feyen, J.H., and Raisz, L.G. 1987. Prostaglandin production by calvariae from sham operated and oophorectomized rats: effect of 17 beta-estradiol in vivo. *Endocrinology*. 121:819-821.

Fibbe, W.E., and Noort, W.A. 2003. Mesenchymal stem cells and hematopoietic stem cell transplantation. *Ann N Y Acad Sci*. 996:235-244.

Filopanti, M., Corbetta, S., Barbieri, A.M., and Spada, A. 2013. Pharmacology of the calcium sensing receptor. *Clin Cases Miner Bone Metab*. 10:162-165.

Forlino, A., Cabral, W.A., Barnes, A.M., and Marini, J.C. 2011. New perspectives on osteogenesis imperfecta. *Nat Rev Endocrinol*. 7:540-557.

Franceschi, R.T., and Iyer, B.S. 1992. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res*. 7:235-246.

Franceschi, R.T., Iyer, B.S., and Cui, Y. 1994. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res*. 9:843-854.

Franceschi, R.T., Wilson, J.X., and Dixon, S.J. 1995. Requirement for Na(+)-dependent ascorbic acid transport in osteoblast function. *Am J Physiol*. 268:C1430-1439.

Franceschi, R.T., and Xiao, G. 2003. Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways. *J Cell Biochem*. 88:446-454.

Friedenstein, A.J., Chailakhjan, R.K., and Lalykina, K.S. 1970. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet*. 3:393-403.

Friedenstein, A.J., Gorskaja, J.F., and Kulagina, N.N. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol*. 4:267-274.

Friedenstein, A.J., Piatetzky, S., II, and Petrakova, K.V. 1966. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol*. 16:381-390.

Frith, J.C., Monkkonen, J., Blackburn, G.M., Russell, R.G., and Rogers, M.J. 1997. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells in vitro. *J Bone Miner Res*. 12:1358-1367.

Fucik, R.F., Kukreja, S.C., Hargis, G.K., Bowser, E.N., Henderson, W.J., and Williams, G.A. 1975. Effect of glucocorticoids on function of the parathyroid glands in man. *J Clin Endocrinol Metab*. 40:152-155.

Fujita, T., Izumo, N., Fukuyama, R., Meguro, T., Nakamuta, H., Kohno, T., and Koida, M. 2001. Phosphate provides an extracellular signal that drives nuclear export of Runx2/Cbfa1 in bone cells. *Biochem Biophys Res Commun*. 280:348-352.

Gao, J., Dennis, J.E., Muzic, R.F., Lundberg, M., and Caplan, A.I. 2001. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs*. 169:12-20.

Gallant, M.A., Brown, D.M., Hammond, M., Wallace, J.M., Du, J., Deymier-Black, A.C., Almer, J.D., Stock, S.R., Allen, M.R., and Burr, D.B. 2014. Bone cell-independent benefits of raloxifene on the skeleton: a novel mechanism for improving bone material properties. *Bone*. 61:191-200.

Gartland, A., Buckley, K.A., Bowler, W.B., and Gallagher, J.A. 2003. Blockade of the pore-forming P2X7 receptor inhibits formation of multinucleated human osteoclasts in vitro. *Calcif Tissue Int*. 73:361-369.

Gartland, A., Hipskind, R.A., Gallagher, J.A., and Bowler, W.B. 2001. Expression of a P2X7 receptor by a subpopulation of human osteoblasts. *J Bone Miner Res*. 16:846-856.

Gartland, A., Skarratt, K.K., Hocking, L.J., Parsons, C., Stokes, L., Jorgensen, N.R., Fraser, W.D., Reid, D.M., Gallagher, J.A., and Wiley, J.S. 2012. Polymorphisms in the P2X7 receptor gene are associated with low lumbar spine bone mineral density and accelerated bone loss in postmenopausal women. *Eur J Hum Genet*. 20:559-564.

Gendron, F.P., Benrezzak, O., Krugh, B.W., Kong, Q., Weisman, G.A., and Beaudoin, A.R. 2002. Purine signalling and potential new therapeutic approach: possible outcomes of NTPDase inhibition. *Curr Drug Targets*. 3:229-245.

Genetos, D.C., Geist, D.J., Liu, D., Donahue, H.J., and Duncan, R.L. 2005. Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. *J Bone Miner Res*. 20:41-49.

Gordon, M.T., Anderson, D.C., and Sharpe, P.T. 1991. Canine distemper virus localised in bone cells of patients with Paget's disease. *Bone*. 12:195-201.

Gordon, M.Y. 1991. Hemopoietic growth factors and receptors: bound and free. *Cancer Cells*. 3:127-133.

Grady, D., Ettinger, B., Moscarelli, E., Plouffe, L., Jr., Sarkar, S., Ciaccia, A., and Cummings, S. 2004. Safety and adverse effects associated with raloxifene: multiple outcomes of raloxifene evaluation. *Obstet Gynecol*. 104:837-844.

Grol, M.W., Panupinthu, N., Korcok, J., Sims, S.M., and Dixon, S.J. 2009. Expression, signalling, and function of P2X7 receptors in bone. *Purinergic Signal*. 5:205-221.

Gronthos, S., Zannettino, A.C., Hay, S.J., Shi, S., Graves, S.E., Kortesidis, A., and Simmons, P.J. 2003. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci*. 116:1827-1835.

Gruener, G., and Camacho, P. 2014. Paget's disease of bone. *Handb Clin Neurol*. 119:529-540.

Gundberg, C.M., Lian, J.B., and Booth, S.L. 2012. Vitamin K-dependent carboxylation of osteocalcin: friend or foe? *Adv Nutr*. 3:149-157.

Hamdy, R.C., Baim, S., Broy, S.B., Lewiecki, E.M., Morgan, S.L., Tanner, S.B., and Williamson, H.F. 2010. Algorithm for the management of osteoporosis. *South Med J*. 103:1009-1015.

Hamilton, C.J., Swan, V.J., and Jamal, S.A. 2010. The effects of exercise and physical activity participation on bone mass and geometry in postmenopausal women: a systematic review of pQCT studies. *Osteoporos Int*. 21:11-23.

Hardy, C.L., and Minguell, J.J. 1995. Modulation of the adhesion of hemopoietic progenitor cells to the RGD site of fibronectin by interleukin 3. *J Cell Physiol*. 164:315-323.

Harley, C.B., Futcher, A.B., and Greider, C.W. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature*. 345:458-460.

Harter, L.V., Hruska, K.A., and Duncan, R.L. 1995. Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. *Endocrinology*. 136:528-535.

Hayton, M.J., Dillon, J.P., Glynn, D., Curran, J.M., Gallagher, J.A., and Buckley, K.A. 2005. Involvement of adenosine 5'-triphosphate in ultrasound-induced fracture repair. *Ultrasound Med Biol*. 31:1131-1138.

Hazama, R., Qu, X., Yokoyama, K., Tanaka, C., Kinoshita, E., He, J., Takahashi, S., Tohyama, K., Yamamura, H., and Tohyama, Y. 2009. ATP-induced osteoclast function: the formation of sealing-zone like structure and the secretion of lytic granules via microtubule-deacetylation under the control of Syk. *Genes Cells*. 14:871-884.

He, W., and Cronstein, B.N. 2012. Adenosine A1 receptor regulates osteoclast formation by altering TRAF6/TAK1 signalling. *Purinergic Signal*. 8:327-337.

Hecht, E., Liedert, A., Ignatius, A., Mizaikoff, B., and Kranz, C. 2013. Local detection of mechanically induced ATP release from bone cells with ATP microbiosensors. *Biosens Bioelectron*. 44:27-33.

Henriksen, Z., Hiken, J.F., Steinberg, T.H., and Jorgensen, N.R. 2006. The predominant mechanism of intercellular calcium wave propagation changes during long-term culture of human osteoblast-like cells. *Cell Calcium*. 39:435-444.

Herbert, J.M., Augereau, J.M., Gleye, J., and Maffrand, J.P. 1990. Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun*. 172:993-999.

Hilton, M.J., Tu, X., Wu, X., Bai, S., Zhao, H., Kobayashi, T., Kronenberg, H.M., Teitelbaum, S.L., Ross, F.P., Kopan, R., and Long, F. 2008. Notch signalling

maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. *Nat Med*. 14:306-314.

Hoebertz, A., Arnett, T.R., and Burnstock, G. 2003. Regulation of bone resorption and formation by purines and pyrimidines. *Trends Pharmacol Sci*. 24:290-297.

Hoebertz, A., Mahendran, S., Burnstock, G., and Arnett, T.R. 2002. ATP and UTP at low concentrations strongly inhibit bone formation by osteoblasts: a novel role for the P2Y2 receptor in bone remodeling. *J Cell Biochem*. 86:413-419.

Hoebertz, A., Meghji, S., Burnstock, G., and Arnett, T.R. 2001. Extracellular ADP is a powerful osteolytic agent: evidence for signalling through the P2Y(1) receptor on bone cells. *FASEB J*. 15:1139-1148.

Hoebertz, A., Townsend-Nicholson, A., Glass, R., Burnstock, G., and Arnett, T.R. 2000. Expression of P2 receptors in bone and cultured bone cells. *Bone*. 27:503-510.

Hoemann, C.D., El-Gabalawy, H., and McKee, M.D. 2009. In vitro osteogenesis assays: influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathol Biol (Paris)*. 57:318-323.

Hofbauer, L.C., Khosla, S., Dunstan, C.R., Lacey, D.L., Spelsberg, T.C., and Riggs, B.L. 1999. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endocrinology*. 140:4367-4370.

Hruska, K.A., Mathew, S., Lund, R., Qiu, P., and Pratt, R. 2008. Hyperphosphatemia of chronic kidney disease. *Kidney Int*. 74:148-157.

Huo, B., Lu, X.L., Hung, C.T., Costa, K.D., Xu, Q., Whitesides, G.M., and Guo, X.E. 2008. Fluid Flow Induced Calcium Response in Bone Cell Network. *Cell Mol Bioeng*. 1:58-66.

Hussar, D.A., and Stevenson, T. 2010. New drugs: Denosumab, dienogest/estradiol valerate, and polidocanol. *J Am Pharm Assoc (2003)*. 50:658-662.

Husted, L.B., Harslof, T., Stenkjaer, L., Carstens, M., Jorgensen, N.R., and Langdahl, B.L. 2013. Functional polymorphisms in the P2X7 receptor gene are associated with osteoporosis. *Osteoporos Int*. 24:949-959.

Ichikawa, J., and Gemba, H. 2009. Cell density-dependent changes in intracellular Ca2+ mobilization via the P2Y2 receptor in rat bone marrow stromal cells. *J Cell Physiol*. 219:372-381.

Ihara, H., Hirukawa, K., Goto, S., and Togari, A. 2005. ATP-stimulated interleukin-6 synthesis through P2Y receptors on human osteoblasts. *Biochem Biophys Res Commun*. 326:329-334.

Ikeda, K., and Takeshita, S. 2014. Factors and mechanisms involved in the coupling from bone resorption to formation: how osteoclasts talk to osteoblasts. *J Bone Metab*. 21:163-167.

Irvin, J.L., and Irvin, E.M. 1954. The interaction of quinacrine with adenine nucleotides. *J Biol Chem*. 210:45-56.

Isern, J., and Mendez-Ferrer, S. 2011. Stem cell interactions in a bone marrow niche. *Curr Osteoporos Rep*. 9:210-218.

Ishikawa, M., Iwamoto, T., Fukumoto, S., and Yamada, Y. 2014. Pannexin 3 inhibits proliferation of osteoprogenitor cells by regulating Wnt and p21 signalling. *J Biol Chem*. 289:2839-2851.

Itoh, T., Nozawa, Y., and Akao, Y. 2009. MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5. *J Biol Chem*. 284:19272-19279.

Iwasaki, H., and Akashi, K. 2007. Hematopoietic developmental pathways: on cellular basis. *Oncogene*. 26:6687-6696.

Jaiswal, N., Haynesworth, S.E., Caplan, A.I., and Bruder, S.P. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem*. 64:295-312.

Janssens, K., ten Dijke, P., Janssens, S., and Van Hul, W. 2005. Transforming growth factor-beta1 to the bone. *Endocr Rev*. 26:743-774.

Jensen, E.D., Gopalakrishnan, R., and Westendorf, J.J. 2010. Regulation of gene expression in osteoblasts. *Biofactors*. 36:25-32.

Johnson, K.A., Hessle, L., Vaingankar, S., Wennberg, C., Mauro, S., Narisawa, S., Goding, J.W., Sano, K., Millan, J.L., and Terkeltaub, R. 2000. Osteoblast tissue-

nonspecific alkaline phosphatase antagonizes and regulates PC-1. *Am J Physiol Regul Integr Comp Physiol*. 279:R1365-1377.

Jones, E.A., Kinsey, S.E., English, A., Jones, R.A., Straszynski, L., Meredith, D.M., Markham, A.F., Jack, A., Emery, P., and McGonagle, D. 2002. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum*. 46:3349-3360.

Jorgensen, N.R., Henriksen, Z., Brot, C., Eriksen, E.F., Sorensen, O.H., Civitelli, R., and Steinberg, T.H. 2000. Human osteoblastic cells propagate intercellular calcium signals by two different mechanisms. *J Bone Miner Res*. 15:1024-1032.

Jorgensen, N.R., Henriksen, Z., Sorensen, O.H., and Civitelli, R. 2004. Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an in vitro model for human bone marrow-derived primary osteoblasts. *Steroids*. 69:219-226.

Jorgensen, N.R., Henriksen, Z., Sorensen, O.H., Eriksen, E.F., Civitelli, R., and Steinberg, T.H. 2002. Intercellular calcium signalling occurs between human osteoblasts and osteoclasts and requires activation of osteoclast P2X7 receptors. *J Biol Chem*. 277:7574-7580.

Jorgensen, N.R., Husted, L.B., Skarratt, K.K., Stokes, L., Tofteng, C.L., Kvist, T., Jensen, J.E., Eiken, P., Brixen, K., Fuller, S., Clifton-Bligh, R., Gartland, A., Schwarz, P., Langdahl, B.L., and Wiley, J.S. 2012. Single-nucleotide polymorphisms in the P2X7 receptor gene are associated with postmenopausal bone loss and vertebral fractures. *Eur J Hum Genet*. 20:675-681.

Joseph, S.M., Pifer, M.A., Przybylski, R.J., and Dubyak, G.R. 2004. Methylene ATP analogs as modulators of extracellular ATP metabolism and accumulation. *Br J Pharmacol*. 142:1002-1014.

Ju, Z., and Lenhard Rudolph, K. 2008. Telomere dysfunction and stem cell ageing. *Biochimie*. 90:24-32.

Jung, Y., Song, J., Shiozawa, Y., Wang, J., Wang, Z., Williams, B., Havens, A., Schneider, A., Ge, C., Franceschi, R.T., McCauley, L.K., Krebsbach, P.H., and Taichman, R.S. 2008. Hematopoietic stem cells regulate mesenchymal stromal

cell induction into osteoblasts thereby participating in the formation of the stem cell niche. *Stem Cells*. 26:2042-2051.

Kandabashi, T., Shimokawa, H., Miyata, K., Kunihiro, I., Eto, Y., Morishige, K., Matsumoto, Y., Obara, K., Nakayama, K., Takahashi, S., and Takeshita, A. 2003. Evidence for protein kinase C-mediated activation of Rho-kinase in a porcine model of coronary artery spasm. *Arterioscler Thromb Vasc Biol*. 23:2209-2214.

Kanematsu, M., Sato, T., Takai, H., Watanabe, K., Ikeda, K., and Yamada, Y. 2000. Prostaglandin E2 induces expression of receptor activator of nuclear factor-kappa B ligand/osteoprotegrin ligand on pre-B cells: implications for accelerated osteoclastogenesis in estrogen deficiency. *J Bone Miner Res*. 15:1321-1329.

Kao, C.F., Chuang, C.Y., Chen, C.H., and Kuo, H.C. 2008. Human pluripotent stem cells: current status and future perspectives. *Chin J Physiol*. 51:214-225.

Kapinas, K., Kessler, C.B., and Delany, A.M. 2009. miR-29 suppression of osteonectin in osteoblasts: regulation during differentiation and by canonical Wnt signalling. *J Cell Biochem*. 108:216-224.

Karaplis, A.C., and Goltzman, D. 2000. PTH and PTHrP effects on the skeleton. *Rev Endocr Metab Disord*. 1:331-341.

Kassem, M., and Marie, P.J. 2011. Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. *Aging Cell*. 10:191-197.

Katz, S., Boland, R., and Santillan, G. 2006. Modulation of ERK 1/2 and p38 MAPK signalling pathways by ATP in osteoblasts: involvement of mechanical stress-activated calcium influx, PKC and Src activation. *Int J Biochem Cell Biol*. 38:2082-2091.

Katz, S., Boland, R., and Santillan, G. 2008. Purinergic (ATP) signalling stimulates JNK1 but not JNK2 MAPK in osteoblast-like cells: contribution of intracellular Ca2+ release, stress activated and L-voltage-dependent calcium influx, PKC and Src kinases. *Arch Biochem Biophys*. 477:244-252.

Kaveh, K., Ibrahim, R., Abu Bakar, M.Z., and Ibrahim, T.A. 2009. Repair of Compact Bone Critical Sized Defect with Natural Originated Scaffold in Rabbit. *J Anim Vet Adv.* 8:1616-1623.

Kaveh, K., Ibrahim, R., Abu Bakar, M.Z., and Ibrahim, T.A. 2011. Mesenchymal Stem Cells, Osteogenic Lineage and Bone Tissue Engineering: A Review. *J Anim Vet Adv.* 10:2317-2330.

Ke, H.Z., Qi, H., Weidema, A.F., Zhang, Q., Panupinthu, N., Crawford, D.T., Grasser, W.A., Paralkar, V.M., Li, M., Audoly, L.P., Gabel, C.A., Jee, W.S., Dixon, S.J., Sims, S.M., and Thompson, D.D. 2003. Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. *Mol Endocrinol*. 17:1356-1367.

Kearns, A.E., Khosla, S., and Kostenuik, P.J. 2008. Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev*. 29:155-192.

Keiding, R., Hörder, M., Denmark, W.G., Pitkänen, E., Tenhunen, R., Strömme, J.H., Theodorsen, L., Waldenström, J., Tryding, N., and Westlund, L. 1974. Recommended methods for the determination of four enzymes in blood. *Scand J Clin Lab Invest*. 33:291-306.

Kern, B., Shen, J., Starbuck, M., and Karsenty, G. 2001. Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. *J Biol Chem*. 276:7101-7107.

King, B.F., Wildman, S.S., Ziganshina, L.E., Pintor, J., and Burnstock, G. 1997. Effects of extracellular pH on agonism and antagonism at a recombinant P2X2 receptor. *Br J Pharmacol*. 121:1445-1453.

Kitazawa, R., Kimble, R.B., Vannice, J.L., Kung, V.T., and Pacifici, R. 1994. Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. *J Clin Invest*. 94:2397-2406.

Koc, O.N., Gerson, S.L., Cooper, B.W., Dyhouse, S.M., Haynesworth, S.E., Caplan, A.I., and Lazarus, H.M. 2000. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow

mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol*. 18:307-316.

Kogawa, M., Anderson, P.H., Findlay, D.M., Morris, H.A., and Atkins, G.J. 2010. The metabolism of 25-(OH)vitamin D3 by osteoclasts and their precursors regulates the differentiation of osteoclasts. *J Steroid Biochem Mol Biol*. 121:277-280.

Kogawa, M., Wijenayaka, A.R., Ormsby, R.T., Thomas, G.P., Anderson, P.H., Bonewald, L.F., Findlay, D.M., and Atkins, G.J. 2013. Sclerostin regulates release of bone mineral by osteocytes by induction of carbonic anhydrase 2. *J Bone Miner Res*. 28:2436-2448.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*. 89:755-764.

Kopen, G.C., Prockop, D.J., and Phinney, D.G. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A*. 96:10711-10716.

Korcok, J., Raimundo, L.N., Du, X., Sims, S.M., and Dixon, S.J. 2005. P2Y6 nucleotide receptors activate NF-kappaB and increase survival of osteoclasts. *J Biol Chem*. 280:16909-16915.

Korcok, J., Raimundo, L.N., Ke, H.Z., Sims, S.M., and Dixon, S.J. 2004. Extracellular nucleotides act through P2X7 receptors to activate NF-kappaB in osteoclasts. *J Bone Miner Res*. 19:642-651.

Kousteni, S., Bellido, T., Plotkin, L.I., O'Brien, C.A., Bodenner, D.L., Han, L., Han, K., DiGregorio, G.B., Katzenellenbogen, J.A., Katzenellenbogen, B.S., Roberson, P.K., Weinstein, R.S., Jilka, R.L., and Manolagas, S.C. 2001. Nongenotropic, sex-nonspecific signalling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell*. 104:719-730.

Kreda, S.M., Okada, S.F., van Heusden, C.A., O'Neal, W., Gabriel, S., Abdullah, L., Davis, C.W., Boucher, R.C., and Lazarowski, E.R. 2007. Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J Physiol*. 584:245-259.

Kreda, S.M., Seminario-Vidal, L., Heusden, C., and Lazarowski, E.R. 2008. Thrombin-promoted release of UDP-glucose from human astrocytoma cells. *Br J Pharmacol*. 153:1528-1537.

Kringelbach, T.M., Aslan, D., Novak, I., Schwarz, P., and Jorgensen, N.R. 2014. UTP-induced ATP release is a fine-tuned signalling pathway in osteocytes. *Purinergic Signal*. 10:337-47.

Kringelbach, T.M., Schwarz, P., Novak, I., Bonewald, L.F., Vang, O., Jørgensen, N.R. 2008. P2 receptors are functionally expressed in MLO-Y4 osteocytes. P*urinergic Signal.* 4:S173–S174.

Kukulski, F., Levesque, S.A., Lavoie, E.G., Lecka, J., Bigonnesse, F., Knowles, A.F., Robson, S.C., Kirley, T.L., and Sevigny, J. 2005. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic Signal*. 1:193-204.

Kukulski, F., Levesque, S.A., and Sevigny, J. 2011. Impact of ectoenzymes on p2 and p1 receptor signalling. *Adv Pharmacol*. 61:263-299.

Kumagai, H., Sacktor, B., and Filburn, C.R. 1991. Purinergic regulation of cytosolic calcium and phosphoinositide metabolism in rat osteoblast-like osteosarcoma cells. *J Bone Miner Res*. 6:697-708.

Landt, M., and Butler, L.G. 1978. 5'-Nucleotide phosphodiesterase: isolation of covalently bound 5'-adenosine monophosphate, an intermediate in the catalytic mechanism. *Biochemistry*. 17:4130-4135.

Lane, J.M., Tomin, E., and Bostrom, M.P. 1999. Biosynthetic bone grafting. *Clin Orthop Relat Res*. 367:S107-117.

Lavoie, E.G., Kukulski, F., Levesque, S.A., Lecka, J., and Sevigny, J. 2004. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-3. *Biochem Pharmacol*. 67:1917-1926.

Lazarowski, E.R., Boucher, R.C., and Harden, T.K. 2000. Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. *J Biol Chem*. 275:31061-31068.

Lazarowski, E.R., Boucher, R.C., and Harden, T.K. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol*. 64:785-795.

Lazarowski, E.R., and Harden, T.K. 1999. Quantitation of extracellular UTP using a sensitive enzymatic assay. *Br J Pharmacol*. 127:1272-1278.

Lazarowski, E.R., Homolya, L., Boucher, R.C., and Harden, T.K. 1997. Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J Biol Chem*. 272:24348-24354.

Lerner, U.H. 2006. Bone remodeling in postmenopausal osteoporosis. *J Dent Res*. 85:584-595.

Leverrier, Y., and Ridley, A.J. 2001. Apoptosis: caspases orchestrate the ROCK 'n' bleb. *Nat Cell Biol*. 3:E91-93.

Li, J., Liu, D., Ke, H.Z., Duncan, R.L., and Turner, C.H. 2005. The P2X7 nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem*. 280:42952-42959.

Li, X., Pilbeam, C.C., Pan, L., Breyer, R.M., and Raisz, L.G. 2002. Effects of prostaglandin E2 on gene expression in primary osteoblastic cells from prostaglandin receptor knockout mice. *Bone*. 30:567-573.

Li, Y.C., Amling, M., Pirro, A.E., Priemel, M., Meuse, J., Baron, R., Delling, G., and Demay, M.B. 1998. Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice. *Endocrinology*. 139:4391-4396.

Li, Z., Hassan, M.Q., Volinia, S., van Wijnen, A.J., Stein, J.L., Croce, C.M., Lian, J.B., and Stein, G.S. 2008. A microRNA signature for a BMP2-induced osteoblast lineage commitment program. *Proc Natl Acad Sci U S A*. 105:13906-13911.

Liang, H., Pun, S., and Wronski, T.J. 1999. Bone anabolic effects of basic fibroblast growth factor in ovariectomized rats. *Endocrinology*. 140:5780-5788.

Liao, J., Hammerick, K.E., Challen, G.A., Goodell, M.A., Kasper, F.K., and Mikos, A.G. 2011. Investigating the role of hematopoietic stem and progenitor cells in regulating the osteogenic differentiation of mesenchymal stem cells in vitro. *J Orthop Res*. 29:1544-1553.

Liu, D., Genetos, D.C., Shao, Y., Geist, D.J., Li, J., Ke, H.Z., Turner, C.H., and Duncan, R.L. 2008b. Activation of extracellular-signal regulated kinase (ERK1/2) by fluid shear is Ca(2+)- and ATP-dependent in MC3T3-E1 osteoblasts. *Bone*. 42:644-652.

Liu, F., Aubin, J.E., and Malaval, L. 2002. Expression of leukemia inhibitory factor (LIF)/interleukin-6 family cytokines and receptors during in vitro osteogenesis: differential regulation by dexamethasone and LIF. *Bone*. 31:212-219.

Liu, H., Toh, W.S., Lu, K., MacAry, P.A., Kemeny, D.M., and Cao, T. 2009. A subpopulation of mesenchymal stromal cells with high osteogenic potential. *J Cell Mol Med*. 13:2436-2447.

Liu, P.S., and Chen, C.Y. 2010. Butyl benzyl phthalate suppresses the ATP-induced cell proliferation in human osteosarcoma HOS cells. *Toxicol Appl Pharmacol*. 244:308-314.

Liu, S.J., Zhang, D.Q., Sui, X.M., Zhang, L., Cai, Z.W., Sun, L.Q., Liu, Y.J., Xue, Y., and Hu, G.F. 2008a. The inhibition of in vivo tumorigenesis of osteosarcoma (OS)-732 cells by antisense human osteopontin RNA. *Cell Mol Biol Lett*. 13:11-19.

Locklin, R.M., Khosla, S., Turner, R.T., and Riggs, B.L. 2003. Mediators of the biphasic responses of bone to intermittent and continuously administered parathyroid hormone. *J Cell Biochem*. 89:180-190.

Lu, X., Alshemali, S., de Wynter, E.A., and Dickinson, A.M. 2010. Mesenchymal stem cells from CD34(-) human umbilical cord blood. *Transfus Med*. 20:178-184.

Luria, E.A., Owen, M.E., Friedenstein, A.J., Morris, J.F., and Kuznetsow, S.A. 1987. Bone formation in organ cultures of bone marrow. *Cell Tissue Res*. 248:449-454.

Ma, Y.L., Cain, R.L., Halladay, D.L., Yang, X., Zeng, Q., Miles, R.R., Chandrasekhar, S., Martin, T.J., and Onyia, J.E. 2001. Catabolic effects of continuous human PTH (1--38) in vivo is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. *Endocrinology*. 142:4047-4054.

Mackenzie, N.C., Zhu, D., Milne, E.M., van 't Hof, R., Martin, A., Darryl Quarles, L., Millan, J.L., Farquharson, C., and MacRae, V.E. 2012. Altered bone development and an increase in FGF-23 expression in Enpp1(-/-) mice. *PLoS One*. 7:e32177.

Magalhães-Cardoso, M.T., Pereira, M.F., Oliveira, L., Ribeiro, J.A., Cunha, R.A., and Correia-de-Sá, P. 2003. Ecto-AMP deaminase blunts the ATP-derived adenosine A2A receptor facilitation of acetylcholine release at rat motor nerve endings. *J Physiol*. 549:399-408.

Maier, R., Glatz, A., Mosbacher, J., and Bilbe, G. 1997. Cloning of P2Y6 cDNAs and identification of a pseudogene: comparison of P2Y receptor subtype expression in bone and brain tissues. *Biochem Biophys Res Commun*. 240:298-302.

Malaval, L., Wade-Gueye, N.M., Boudiffa, M., Fei, J., Zirngibl, R., Chen, F., Laroche, N., Roux, J.P., Burt-Pichat, B., Duboeuf, F., Boivin, G., Jurdic, P., Lafage-Proust, M.H., Amedee, J., Vico, L., Rossant, J., and Aubin, J.E. 2008. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. *J Exp Med*. 205:1145-1153.

Malluche, H.H., Monier-Faugere, M.C., and Herberth, J. 2010. Bone disease after renal transplantation. *Nat Rev Nephrol*. 6:32-40.

Mamedova, L.K., Joshi, B.V., Gao, Z.G., von Kugelgen, I., and Jacobson, K.A. 2004. Diisothiocyanate derivatives as potent, insurmountable antagonists of P2Y6 nucleotide receptors. *Biochem Pharmacol*. 67:1763-1770.

Manolagas, S.C. 2010. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocr Rev*. 31:266-300.

Manolagas, S.C., and Jilka, R.L. 1992. Cytokines, hematopoiesis, osteoclastogenesis, and estrogens. *Calcif Tissue Int*. 50:199-202.

Manolagas, S.C., Kousteni, S., and Jilka, R.L. 2002. Sex steroids and bone. *Recent Prog Horm Res*. 57:385-409.

Marie, P.J., Ammann, P., Boivin, G., and Rey, C. 2001. Mechanisms of action and therapeutic potential of strontium in bone. *Calcif Tissue Int*. 69:121-129.

Masi, L., and Brandi, M.L. 2007. Calcitonin and calcitonin receptors. *Clin Cases Miner Bone Metab*. 4:117-122.

Mateo, J., Harden, T.K., and Boyer, J.L. 1999. Functional expression of a cDNA encoding a human ecto-ATPase. *Br J Pharmacol*. 128:396-402.

Matsuoka, I., and Ohkubo, S. 2004. ATP- and adenosine-mediated signalling in the central nervous system: adenosine receptor activation by ATP through rapid and localized generation of adenosine by ecto-nucleotidases. *J Pharmacol Sci*. 94:95-99.

McBeath, R., Pirone, D.M., Nelson, C.M., Bhadriraju, K., and Chen, C.S. 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell*. 6:483-495.

Mediero, A., Kara, F.M., Wilder, T., and Cronstein, B.N. 2012. Adenosine A(2A) receptor ligation inhibits osteoclast formation. *Am J Pathol*. 180:775-786.

Milovanovic, P., Zimmermann, E.A., Hahn, M., Djonic, D., Puschel, K., Djuric, M., Amling, M., and Busse, B. 2013. Osteocytic canalicular networks: morphological implications for altered mechanosensitivity. *ACS Nano*. 7:7542-7551.

Misiorowski, W. 2011. Parathyroid hormone and its analogues--molecular mechanisms of action and efficacy in osteoporosis therapy. *Endokrynol Pol*. 62:73-78.

Mitalipov, S., and Wolf, D. 2009. Totipotency, pluripotency and nuclear reprogramming. *Adv Biochem Eng Biotechnol*. 114:185-199.

Miyazaki, T., Iwasawa, M., Nakashima, T., Mori, S., Shigemoto, K., Nakamura, H., Katagiri, H., Takayanagi, H., and Tanaka, S. 2012. Intracellular and extracellular ATP coordinately regulate the inverse correlation between osteoclast survival and bone resorption. *J Biol Chem*. 287:37808-37823.

Mizuno, M., and Kuboki, Y. 2001. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. *J Biochem*. 129:133-138.

Moe, S., Drueke, T., Cunningham, J., Goodman, W., Martin, K., Olgaard, K., Ott, S., Sprague, S., Lameire, N., and Eknoyan, G. 2006. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int*. 69:1945-1953.

Moe, S.M. 2008. Disorders involving calcium, phosphorus, and magnesium. *Prim Care*. 35:215-237.

Montero, A., Okada, Y., Tomita, M., Ito, M., Tsurukami, H., Nakamura, T., Doetschman, T., Coffin, J.D., and Hurley, M.M. 2000. Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. *J Clin Invest*. 105:1085-1093.

Moore, E.E., Kuestner, R.E., Stroop, S.D., Grant, F.J., Matthewes, S.L., Brady, C.L., Sexton, P.M., and Findlay, D.M. 1995. Functionally different isoforms of the human calcitonin receptor result from alternative splicing of the gene transcript. *Mol Endocrinol*. 9:959-968.

Moore, W.R., Graves, S.E., and Bain, G.I. 2001. Synthetic bone graft substitutes. *ANZ J Surg*. 71:354-361.

Morelli, A., Chiozzi, P., Chiesa, A., Ferrari, D., Sanz, J.M., Falzoni, S., Pinton, P., Rizzuto, R., Olson, M.F., and Di Virgilio, F. 2003. Extracellular ATP causes ROCK I-dependent bleb formation in P2X7-transfected HEK293 cells. *Mol Biol Cell*. 14:2655-2664.

Morohashi, T., Sano, T., Harai, K., and Yamada, S. 1995. Effects of strontium on calcium metabolism in rats. II. Strontium prevents the increased rate of bone turnover in ovariectomized rats. *Jpn J Pharmacol*. 68:153-159.

Morris, H.A., and Anderson, P.H. 2010. Autocrine and paracrine actions of vitamin d. *Clin Biochem Rev*. 31:129-138.

Morrison, M.S., Turin, L., King, B.F., Burnstock, G., and Arnett, T.R. 1998. ATP is a potent stimulator of the activation and formation of rodent osteoclasts. *J Physiol*. 511:495-500.

Morrison, S.J., Prowse, K.R., Ho, P., and Weissman, I.L. 1996. Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity*. 5:207-216.

Muguruma, Y., Yahata, T., Miyatake, H., Sato, T., Uno, T., Itoh, J., Kato, S., Ito, M., Hotta, T., and Ando, K. 2006. Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood*. 107:1878-1887.

Mundlos, S., Otto, F., Mundlos, C., Mulliken, J.B., Aylsworth, A.S., Albright, S., Lindhout, D., Cole, W.G., Henn, W., Knoll, J.H., Owen, M.J., Mertelsmann, R., Zabel, B.U., and Olsen, B.R. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell*. 89:773-779.

Munkonda, M.N., Pelletier, J., Ivanenkov, V.V., Fausther, M., Tremblay, A., Kunzli, B., Kirley, T.L., and Sevigny, J. 2009. Characterization of a monoclonal antibody as the first specific inhibitor of human NTP diphosphohydrolase-3: partial characterization of the inhibitory epitope and potential applications. *FEBS J*. 276:479-496.

Naemsch, L.N., Dixon, S.J., and Sims, S.M. 2001. Activity-dependent development of P2X7 current and Ca2+ entry in rabbit osteoclasts. *J Biol Chem*. 276:39107-39114.

Nakamura, E., Uezono, Y., Narusawa, K., Shibuya, I., Oishi, Y., Tanaka, M., Yanagihara, N., Nakamura, T., and Izumi, F. 2000. ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells. *Am J Physiol Cell Physiol*. 279:C510-519.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R., and de Crombrugghe, B. 2002. The novel zinc finger-containing transcription factor

osterix is required for osteoblast differentiation and bone formation. *Cell*. 108:17-29.

Neuprez, A., Hiligsmann, M., Scholtissen, S., Bruyere, O., and Reginster, J.Y. 2008. Strontium ranelate: the first agent of a new therapeutic class in osteoporosis. *Adv Ther*. 25:1235-1256.

Nicotera, P., Hartzell, P., Davis, G., and Orrenius, S. 1986. The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca2+ is mediated by the activation of a non-lysosomal proteolytic system. *FEBS Lett*. 209:139-144.

Nitschke, Y., Baujat, G., Botschen, U., Wittkampf, T., du Moulin, M., Stella, J., Le Merrer, M., Guest, G., Lambot, K., Tazarourte-Pinturier, M.F., Chassaing, N., Roche, O., Feenstra, I., Loechner, K., Deshpande, C., Garber, S.J., Chikarmane, R., Steinmann, B., Shahinyan, T., Martorell, L., Davies, J., Smith, W.E., Kahler, S.G., McCulloch, M., Wraige, E., Loidi, L., Hohne, W., Martin, L., Hadj-Rabia, S., Terkeltaub, R., and Rutsch, F. 2012. Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCC6. *Am J Hum Genet*. 90:25-39.

Norman, A.W. 2008. From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am J Clin Nutr*. 88:491S-499S.

Noronha-Matos, J.B., Coimbra, J., Sá-e-Sousa, A., Rocha, R., Marinhas, J., Freitas, R., Guerra-Gomes, S., Ferreirinha, F., Costa, M.A., and Correia-de-Sá, P. 2014. P2X7-induced zeiosis promotes osteogenic differentiation and mineralization of postmenopausal bone marrow-derived mesenchymal stem cells. *FASEB J*. Epub ahead of print. pii: fj.14-257923.

Noronha-Matos, J.B., Costa, M.A., Magalhaes-Cardoso, M.T., Ferreirinha, F., Pelletier, J., Freitas, R., Neves, J.M., Sevigny, J., and Correia-de-Sá, P. 2012. Role of ecto-NTPDases on UDP-sensitive P2Y(6) receptor activation during osteogenic differentiation of primary bone marrow stromal cells from postmenopausal women. *J Cell Physiol*. 227:2694-2709.

North, R.A. 2002. Molecular physiology of P2X receptors. *Physiol Rev*. 82:1013-1067.

Novak, I. 2003. ATP as a signalling molecule: the exocrine focus. *News Physiol Sci*. 18:12-17.

Nugent, M.A., and Iozzo, R.V. 2000. Fibroblast growth factor-2. *Int J Biochem Cell Biol*. 32:115-120.

Nussenzveig, D.R., Thaw, C.N., and Gershengorn, M.C. 1994. Inhibition of inositol phosphate second messenger formation by intracellular loop one of a human calcitonin receptor. Expression and mutational analysis of synthetic receptor genes. *J Biol Chem*. 269:28123-28129.

Nuttelman, C.R., Tripodi, M.C., and Anseth, K.S. 2005. Synthetic hydrogel niches that promote hMSC viability. *Matrix Biol*. 24:208-218.

Ohlendorff, S.D., Tofteng, C.L., Jensen, J.E., Petersen, S., Civitelli, R., Fenger, M., Abrahamsen, B., Hermann, A.P., Eiken, P., and Jorgensen, N.R. 2007. Single nucleotide polymorphisms in the P2X7 gene are associated to fracture risk and to effect of estrogen treatment. *Pharmacogenet Genomics*. 17:555-567.

Ono, K., Kaneko, H., Choudhary, S., Pilbeam, C.C., Lorenzo, J.A., Akatsu, T., Kugai, N., and Raisz, L.G. 2005. Biphasic effect of prostaglandin E2 on osteoclast formation in spleen cell cultures: role of the EP2 receptor. *J Bone Miner Res*. 20:23-29.

Orriss, I., Syberg, S., Wang, N., Robaye, B., Gartland, A., Jorgensen, N., Arnett, T., and Boeynaems, J.M. 2011. Bone phenotypes of P2 receptor knockout mice. *Front Biosci (Schol Ed)*. 3:1038-1046.

Orriss, I.R., Burnstock, G., and Arnett, T.R. 2010. Purinergic signalling and bone remodelling. *Curr Opin Pharmacol*. 10:322-330.

Orriss, I.R., Key, M.L., Brandao-Burch, A., Patel, J.J., Burnstock, G., and Arnett, T.R. 2012. The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of p2x receptors. *Bone*. 51:389-400.

Orriss, I.R., Key, M.L., Hajjawi, M.O., and Arnett, T.R. 2013. Extracellular ATP released by osteoblasts is a key local inhibitor of bone mineralisation. *PLoS One*. 8:e69057.

Orriss, I.R., Knight, G.E., Ranasinghe, S., Burnstock, G., and Arnett, T.R. 2006. Osteoblast responses to nucleotides increase during differentiation. *Bone*. 39:300-309.

Orriss, I.R., Knight, G.E., Utting, J.C., Taylor, S.E., Burnstock, G., and Arnett, T.R. 2009. Hypoxia stimulates vesicular ATP release from rat osteoblasts. *J Cell Physiol*. 220:155-162.

Orriss, I.R., Utting, J.C., Brandao-Burch, A., Colston, K., Grubb, B.R., Burnstock, G., and Arnett, T.R. 2007. Extracellular nucleotides block bone mineralization in vitro: evidence for dual inhibitory mechanisms involving both P2Y2 receptors and pyrophosphate. *Endocrinology*. 148:4208-4216.

Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., Selby, P.B., and Owen, M.J. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*. 89:765-771.

Oursler, M.J., Cortese, C., Keeting, P., Anderson, M.A., Bonde, S.K., Riggs, B.L., and Spelsberg, T.C. 1991. Modulation of transforming growth factor-beta production in normal human osteoblast-like cells by 17 beta-estradiol and parathyroid hormone. *Endocrinology*. 129:3313-3320.

Owen, T.A., Bortell, R., Yocum, S.A., Smock, S.L., Zhang, M., Abate, C., Shalhoub, V., Aronin, N., Wright, K.L., van Wijnen, A.J., Stein, J.L., Curran, T., Lian, J.B., and Stein, G. 1990. Coordinate occupancy of AP-1 sites in the vitamin D-responsive and CCAAT box elements by Fos-Jun in the osteocalcin gene: model for phenotype suppression of transcription. *Proc Natl Acad Sci U S A*. 87:9990-9994.

Pacifici, R., Brown, C., Puscheck, E., Friedrich, E., Slatopolsky, E., Maggio, D., McCracken, R., and Avioli, L.V. 1991. Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc Natl Acad Sci U S A*. 88:5134-5138.

Panupinthu, N., Rogers, J.T., Zhao, L., Solano-Flores, L.P., Possmayer, F., Sims, S.M., and Dixon, S.J. 2008. P2X7 receptors on osteoblasts couple to production

of lysophosphatidic acid: a signalling axis promoting osteogenesis. *J Cell Biol*. 181:859-871.

Panupinthu, N., Zhao, L., Possmayer, F., Ke, H.Z., Sims, S.M., and Dixon, S.J. 2007. P2X7 nucleotide receptors mediate blebbing in osteoblasts through a pathway involving lysophosphatidic acid. *J Biol Chem*. 282:3403-3412.

Paredes, R., Arriagada, G., Cruzat, F., Olate, J., Van Wijnen, A., Lian, J., Stein, G., Stein, J., and Montecino, M. 2004. The Runx2 transcription factor plays a key role in the 1alpha,25-dihydroxy Vitamin D3-dependent upregulation of the rat osteocalcin (OC) gene expression in osteoblastic cells. *J Steroid Biochem Mol Biol*. 89-90:269-271.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D., and Lapidot, T. 1999. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science*. 283:845-848.

Pelegrin, P., and Surprenant, A. 2006. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J*. 25:5071-5082.

Pellegatti, P., Falzoni, S., Donvito, G., Lemaire, I., and Di Virgilio, F. 2011. P2X7 receptor drives osteoclast fusion by increasing the extracellular adenosine concentration. *FASEB J*. 25:1264-1274.

Pfeilschifter, J., Diel, I., Pilz, U., Brunotte, K., Naumann, A., and Ziegler, R. 1993. Mitogenic responsiveness of human bone cells in vitro to hormones and growth factors decreases with age. *J Bone Miner Res*. 8:707-717.

Picher, M., Sevigny, J., D'Orleans-Juste, P., and Beaudoin, A.R. 1996. Hydrolysis of P2-purinoceptor agonists by a purified ectonucleotidase from the bovine aorta, the ATP-diphosphohydrolase. *Biochem Pharmacol*. 51:1453-1460.

Pignolo, R.J., Suda, R.K., McMillan, E.A., Shen, J., Lee, S.H., Choi, Y., Wright, A.C., and Johnson, F.B. 2008. Defects in telomere maintenance molecules impair osteoblast differentiation and promote osteoporosis. *Aging Cell*. 7:23-31.

Pines, A., Romanello, M., Cesaratto, L., Damante, G., Moro, L., D'Andrea, P., and Tell, G. 2003. Extracellular ATP stimulates the early growth response protein 1

(Egr-1) via a protein kinase C-dependent pathway in the human osteoblastic HOBIT cell line. *Biochem J*. 373:815-824.

Pinheiro, A.R., Paramos-de-Carvalho, D., Certal, M., Costa, C., Magalhães-Cardoso, M.T., Ferreirinha, F., Costa, M.A., and Correia-de-Sá, P. 2013. Bradykinin-induced Ca2+ signalling in human subcutaneous fibroblasts involves ATP release via hemichannels leading to P2Y12 receptors activation. *Cell Commun Signal*. 11:70.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science*. 284:143-147.

Plotkin, L.I., Manolagas, S.C., and Bellido, T. 2002. Transduction of cell survival signals by connexin-43 hemichannels. *J Biol Chem*. 277:8648-8657.

Pondel, M. 2000. Calcitonin and calcitonin receptors: bone and beyond. *Int J Exp Pathol*. 81:405-422.

Porada, C.D., Zanjani, E.D., and Almeida-Porad, G. 2006. Adult mesenchymal stem cells: a pluripotent population with multiple applications. *Curr Stem Cell Res Ther*. 1:365-369.

Qu, Q., Perala-Heape, M., Kapanen, A., Dahllund, J., Salo, J., Vaananen, H.K., and Harkonen, P. 1998. Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. *Bone*. 22:201-209.

Radeff, J.M., Nagy, Z., and Stern, P.H. 2004. Rho and Rho kinase are involved in parathyroid hormone-stimulated protein kinase C alpha translocation and IL-6 promoter activity in osteoblastic cells. *J Bone Miner Res*. 19:1882-1891.

Raggi, C., and Berardi, A.C. 2012. Mesenchymal stem cells, aging and regenerative medicine. *Muscles Ligaments Tendons J*. 2:239-242.

Raisz, L.G. 1999. Physiology and pathophysiology of bone remodeling. *Clin Chem*. 45:1353-1358.

Ralevic, V., and Burnstock, G. 1998. Receptors for purines and pyrimidines. *Pharmacol Rev*. 50:413-492.

Rando, T.A. 2006. Stem cells, ageing and the quest for immortality. *Nature*. 441:1080-1086.

Rang, H.P., Dale, M.M., Ritter, J.M., Flower, R.J., and Henderson G. 2011. Rang and Dale's pharmacology. *7th edition Elsevier/Churchill Livingstone, Edinburgh; New York.* 35: 432-441.

Rauch, F., Munns, C., Land, C., and Glorieux, F.H. 2006. Pamidronate in children and adolescents with osteogenesis imperfecta: effect of treatment discontinuation. *J Clin Endocrinol Metab*. 91:1268-1274.

Reichel, H., Koeffler, H.P., and Norman, A.W. 1989. The role of the vitamin D endocrine system in health and disease. *N Engl J Med*. 320:980-991.

Riddle, R.C., Taylor, A.F., Rogers, J.R., and Donahue, H.J. 2007. ATP release mediates fluid flow-induced proliferation of human bone marrow stromal cells. *J Bone Miner Res*. 22:589-600.

Robson, S.C., Sevigny, J., and Zimmermann, H. 2006. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal*. 2:409-430.

Roger, S., Gillet, L., Baroja-Mazo, A., Surprenant, A., and Pelegrin, P. 2010. C-terminal calmodulin-binding motif differentially controls human and rat P2X7 receptor current facilitation. *J Biol Chem*. 285:17514-17524.

Rohrbach, M., and Giunta, C. 2012. Recessive osteogenesis imperfecta: clinical, radiological, and molecular findings. *Am J Med Genet C Semin Med Genet*. 160C:175-189.

Romanello, M., Bivi, N., Pines, A., Deganuto, M., Quadrifoglio, F., Moro, L., and Tell, G. 2006. Bisphosphonates activate nucleotide receptors signalling and induce the expression of Hsp90 in osteoblast-like cell lines. *Bone*. 39:739-753.

Romanello, M., Codognotto, A., Bicego, M., Pines, A., Tell, G., and D'Andrea, P. 2005. Autocrine/paracrine stimulation of purinergic receptors in osteoblasts: contribution of vesicular ATP release. *Biochem Biophys Res Commun*. 331:1429-1438.

Romanello, M., Pani, B., Bicego, M., and D'Andrea, P. 2001. Mechanically induced ATP release from human osteoblastic cells. *Biochem Biophys Res Commun*. 289:1275-1281.

Rumney, R.M., Sunters, A., Reilly, G.C., and Gartland, A. 2012. Application of multiple forms of mechanical loading to human osteoblasts reveals increased ATP release in response to fluid flow in 3D cultures and differential regulation of immediate early genes. *J Biomech*. 45:549-554.

Rutsch, F., Ruf, N., Vaingankar, S., Toliat, M.R., Suk, A., Hohne, W., Schauer, G., Lehmann, M., Roscioli, T., Schnabel, D., Epplen, J.T., Knisely, A., Superti-Furga, A., McGill, J., Filippone, M., Sinaiko, A.R., Vallance, H., Hinrichs, B., Smith, W., Ferre, M., Terkeltaub, R., and Nurnberg, P. 2003. Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification. *Nat Genet*. 34:379-381.

Sabirov, R.Z., and Okada, Y. 2009. The maxi-anion channel: a classical channel playing novel roles through an unidentified molecular entity. *J Physiol Sci*. 59:3-21.

Saeed, H., Abdallah, B.M., Ditzel, N., Catala-Lehnen, P., Qiu, W., Amling, M., and Kassem, M. 2011. Telomerase-deficient mice exhibit bone loss owing to defects in osteoblasts and increased osteoclastogenesis by inflammatory microenvironment. *J Bone Miner Res*. 26:1494-1505.

Saidak, Z., and Marie, P.J. 2012. Strontium signalling: molecular mechanisms and therapeutic implications in osteoporosis. *Pharmacol Ther*. 136:216-226.

Sasaki, Y., Suzuki, M., and Hidaka, H. 2002. The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. *Pharmacol Ther*. 93:225-232.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A.H. 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signalling by a pharmacological GSK-3-specific inhibitor. *Nat Med*. 10:55-63.

Schmidt, C., Pommerenke, H., Durr, F., Nebe, B., and Rychly, J. 1998. Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. *J Biol Chem*. 273:5081-5085.

Schmidt, R., and Kulbe, K.D. 1993. Long-term cultivation of human osteoblasts. *Bone Miner*. 20:211-221.

Schuiling, K.D., Robinia, K., and Nye, R. 2011. Osteoporosis update. *J Midwifery Womens Health*. 56:615-627.

Selvamurugan, N., Chou, W.Y., Pearman, A.T., Pulumati, M.R., and Partridge, N.C. 1998. Parathyroid hormone regulates the rat collagenase-3 promoter in osteoblastic cells through the cooperative interaction of the activator protein-1 site and the runt domain binding sequence. *J Biol Chem*. 273:10647-10657.

Sfeir, A.J., Chai, W., Shay, J.W., and Wright, W.E. 2005. Telomere-end processing the terminal nucleotides of human chromosomes. *Mol Cell*. 18:131-138.

Shake, J.G., Gruber, P.J., Baumgartner, W.A., Senechal, G., Meyers, J., Redmond, J.M., Pittenger, M.F., and Martin, B.J. 2002. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg*. 73:1919-1925.

Shay, J.W., and Wright, W.E. 2000. Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol*. 1:72-76.

Shestopalov, V.I., and Panchin, Y. 2008. Pannexins and gap junction protein diversity. *Cell Mol Life Sci*. 65:376-394.

Simmons, P.J., Zannettino, A., Gronthos, S., and Leavesley, D. 1994. Potential adhesion mechanisms for localisation of haemopoietic progenitors to bone marrow stroma. *Leuk Lymphoma*. 12:353-363.

Simonsen, J.L., Rosada, C., Serakinci, N., Justesen, J., Stenderup, K., Rattan, S.I., Jensen, T.G., and Kassem, M. 2002. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. *Nat Biotechnol*. 20:592-596.

Singer, F.R. 2009. Paget disease: when to treat and when not to treat. *Nat Rev Rheumatol*. 5:483-489.

Sinha, K.M., and Zhou, X. 2013. Genetic and molecular control of osterix in skeletal formation. *J Cell Biochem*. 114:975-984.

Siris, E.S. 1995. Extensive personal experience: Paget's disease of bone. *J Clin Endocrinol Metab*. 80:335-338.

Sivasubramaniyan, K., Lehnen, D., Ghazanfari, R., Sobiesiak, M., Harichandan, A., Mortha, E., Petkova, N., Grimm, S., Cerabona, F., de Zwart, P., Abele, H., Aicher, W.K., Faul, C., Kanz, L., and Buhring, H.J. 2012. Phenotypic and functional heterogeneity of human bone marrow- and amnion-derived MSC subsets. *Ann N Y Acad Sci*. 1266:94-106.

Sly, W.S., and Hu, P.Y. 1995. Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu Rev Biochem*. 64:375-401.

Smith, T.M., and Kirley, T.L. 1999. Site-directed mutagenesis of a human brain ecto-apyrase: evidence that the E-type ATPases are related to the actin/heat shock 70/sugar kinase superfamily. *Biochemistry*. 38:321-328.

Sneddon, W.B., Magyar, C.E., Willick, G.E., Syme, C.A., Galbiati, F., Bisello, A., and Friedman, P.A. 2004. Ligand-selective dissociation of activation and internalization of the parathyroid hormone (PTH) receptor: conditional efficacy of PTH peptide fragments. *Endocrinology*. 145:2815-2823.

Soulet, C., Hechler, B., Gratacap, M.P., Plantavid, M., Offermanns, S., Gachet, C., and Payrastre, B. 2005. A differential role of the platelet ADP receptors P2Y1 and P2Y12 in Rac activation. *J Thromb Haemost*. 3:2296-2306.

Sovenyhazy, K.M., Bordelon, J.A., and Petty, J.T. 2003. Spectroscopic studies of the multiple binding modes of a trimethine-bridged cyanine dye with DNA. *Nucleic Acids Res*. 31:2561-2569.

Stefan, C., Jansen, S., and Bollen, M. 2005. NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem Sci*. 30:542-550.

Stenderup, K., Justesen, J., Clausen, C., and Kassem, M. 2003. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone*. 33:919-926.

Stoicov, C., Li, H., Liu, J.H., and Houghton, J. 2013. Mesenchymal stem cells utilize CXCR4-SDF-1 signalling for acute, but not chronic, trafficking to gastric mucosal inflammation. *Dig Dis Sci*. 58:2466-2477.

Su, X., Floyd, D.H., Hughes, A., Xiang, J., Schneider, J.G., Uluckan, O., Heller, E., Deng, H., Zou, W., Craft, C.S., Wu, K., Hirbe, A.C., Grabowska, D., Eagleton, M.C., Townsley, S., Collins, L., Piwnica-Worms, D., Steinberg, T.H., Novack, D.V., Conley, P.B., Hurchla, M.A., Rogers, M., and Weilbaecher, K.N. 2012. The ADP receptor P2RY12 regulates osteoclast function and pathologic bone remodeling. *J Clin Invest*. 122:3579-3592.

Sudo, K., Ema, H., Morita, Y., and Nakauchi, H. 2000. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med*. 192:1273-1280.

Sun, D., Junger, W.G., Yuan, C., Zhang, W., Bao, Y., Qin, D., Wang, C., Tan, L., Qi, B., Zhu, D., Zhang, X., and Yu, T. 2013. Shockwaves induce osteogenic differentiation of human mesenchymal stem cells through ATP release and activation of P2X7 receptors. *Stem Cells*. 31:1170-1180.

Swarthout, J.T., D'Alonzo, R.C., Selvamurugan, N., and Partridge, N.C. 2002. Parathyroid hormone-dependent signalling pathways regulating genes in bone cells. *Gene*. 282:1-17.

Syberg, S., Brandao-Burch, A., Patel, J.J., Hajjawi, M., Arnett, T.R., Schwarz, P., Jorgensen, N.R., and Orriss, I.R. 2012. Clopidogrel (Plavix), a P2Y12 receptor antagonist, inhibits bone cell function in vitro and decreases trabecular bone in vivo. *J Bone Miner Res*. 27:2373-2386.

Taichman, R.S. 2005. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood*. 105:2631-2639.

Takahashi, N., Akatsu, T., Sasaki, T., Nicholson, G.C., Moseley, J.M., Martin, T.J., and Suda, T. 1988. Induction of calcitonin receptors by 1 alpha, 25-dihydroxyvitamin D3 in osteoclast-like multinucleated cells formed from mouse bone marrow cells. *Endocrinology*. 123:1504-1510.

Takarada, T., Hinoi, E., Nakazato, R., Ochi, H., Xu, C., Tsuchikane, A., Takeda, S., Karsenty, G., Abe, T., Kiyonari, H., and Yoneda, Y. 2013. An analysis of skeletal development in osteoblast-specific and chondrocyte-specific runt-

related transcription factor-2 (Runx2) knockout mice. *J Bone Miner Res*. 28:2064-2069.

Takata, S., and Yasui, N. 2001. Disuse osteoporosis. *J Med Invest*. 48:147-156.

Takedachi, M., Oohara, H., Smith, B.J., Iyama, M., Kobashi, M., Maeda, K., Long, C.L., Humphrey, M.B., Stoecker, B.J., Toyosawa, S., Thompson, L.F., and Murakami, S. 2012. CD73-generated adenosine promotes osteoblast differentiation. *J Cell Physiol*. 227:2622-2631.

Takuma, A., Kaneda, T., Sato, T., Ninomiya, S., Kumegawa, M., and Hakeda, Y. 2003. Dexamethasone enhances osteoclast formation synergistically with transforming growth factor-beta by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts. *J Biol Chem*. 278:44667-44674.

Tamura, M., Nakao, H., Yoshizaki, H., Shiratsuchi, M., Shigyo, H., Yamada, H., Ozawa, T., Totsuka, J., and Hidaka, H. 2005. Development of specific Rho-kinase inhibitors and their clinical application. *Biochim Biophys Acta*. 1754:245-252.

Tateishi, K., Ando, W., Higuchi, C., Hart, D.A., Hashimoto, J., Nakata, K., Yoshikawa, H., and Nakamura, N. 2008. Comparison of human serum with fetal bovine serum for expansion and differentiation of human synovial MSC: potential feasibility for clinical applications. *Cell Transplant*. 17:549-557.

Tatsumi, S., Ishii, K., Amizuka, N., Li, M., Kobayashi, T., Kohno, K., Ito, M., Takeshita, S., and Ikeda, K. 2007. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab*. 5:464-475.

Teitelbaum, S.L. 1993. Molecular defects of Bone Development*. In* Primer *on the* Metabolic Bone Diseases and Disorders of Mineral Metabolism*.* 2nd edition. *Favus M., J. Raven Press. New York.* 2:10-15.

Tella, S.H., and Gallagher, J.C. 2014. Prevention and treatment of postmenopausal osteoporosis. *J Steroid Biochem Mol Biol*. 142:155-170.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., and Dawson, A.P. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. *Proc Natl Acad Sci U S A*. 87:2466-2470.

Thi, M.M., Islam, S., Suadicani, S.O., and Spray, D.C. 2012. Connexin43 and pannexin1 channels in osteoblasts: who is the "hemichannel"? *J Membr Biol*. 245:401-409.

Thompson, W.R., Majid, A.S., Czymmek, K.J., Ruff, A.L., Garcia, J., Duncan, R.L., and Farach-Carson, M.C. 2011. Association of the alpha(2)delta(1) subunit with Ca(v)3.2 enhances membrane expression and regulates mechanically induced ATP release in MLO-Y4 osteocytes. *J Bone Miner Res*. 26:2125-2139.

Toksoz, D., Zsebo, K.M., Smith, K.A., Hu, S., Brankow, D., Suggs, S.V., Martin, F.H., and Williams, D.A. 1992. Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. *Proc Natl Acad Sci U S A*. 89:7350-7354.

Toraldo, G., Roggia, C., Qian, W.P., Pacifici, R., and Weitzmann, M.N. 2003. IL-7 induces bone loss in vivo by induction of receptor activator of nuclear factor kappa B ligand and tumor necrosis factor alpha from T cells. *Proc Natl Acad Sci U S A*. 100:125-130.

Trombetta, E.S., and Helenius, A. 1999. Glycoprotein reglucosylation and nucleotide sugar utilization in the secretory pathway: identification of a nucleoside diphosphatase in the endoplasmic reticulum. *EMBO J*. 18:3282-3292.

Tumpel, S., and Rudolph, K.L. 2012. The role of telomere shortening in somatic stem cells and tissue aging: lessons from telomerase model systems. *Ann N Y Acad Sci*. 1266:28-39.

Turner, R.T., Evans, G.L., and Wakley, G.K. 1993. Mechanism of action of estrogen on cancellous bone balance in tibiae of ovariectomized growing rats: inhibition of indices of formation and resorption. *J Bone Miner Res*. 8:359-366.

van Staa, T.P., Leufkens, H.G., Abenhaim, L., Zhang, B., and Cooper, C. 2000. Oral corticosteroids and fracture risk: relationship to daily and cumulative doses. *Rheumatology (Oxford)*. 39:1383-1389.

Varenna, M., Bertoldo, F., Di Monaco, M., Giusti, A., Martini, G., and Rossini, M. 2013. Safety profile of drugs used in the treatment of osteoporosis: a systematical review of the literature. *Reumatismo*. 65:143-166.

Vats, A., Tolley, N.S., Polak, J.M., and Gough, J.E. 2003. Scaffolds and biomaterials for tissue engineering: a review of clinical applications. *Clin Otolaryngol Allied Sci*. 28:165-172.

Verburg, F.A., Reiners, C., Grelle, I., Barth, H., Fassnacht, M., and Luster, M. 2013. Calcium stimulated calcitonin measurement: a procedural proposal. *Exp Clin Endocrinol Diabetes*. 121:318-320.

Verfaillie, C.M. 1998. Adhesion receptors as regulators of the hematopoietic process. *Blood*. 92:2609-2612.

Verhoef, P.A., Estacion, M., Schilling, W., and Dubyak, G.R. 2003. P2X7 receptor-dependent blebbing and the activation of Rho-effector kinases, caspases, and IL-1 beta release. *J Immunol*. 170:5728-5738.

Volarevic, V., Al-Qahtani, A., Arsenijevic, N., Pajovic, S., and Lukic, M.L. 2010. Interleukin-1 receptor antagonist (IL-1Ra) and IL-1Ra producing mesenchymal stem cells as modulators of diabetogenesis. *Autoimmunity*. 43:255-263.

Volarevic, V., Ljujic, B., Stojkovic, P., Lukic, A., Arsenijevic, N., and Stojkovic, M. 2011. Human stem cell research and regenerative medicine-present and future. *Br Med Bull*. 99:155-168.

Volonte, C., and D'Ambrosi, N. 2009. Membrane compartments and purinergic signalling: the purinome, a complex interplay among ligands, degrading enzymes, receptors and transporters. *FEBS J*. 276:318-329.

von Zglinicki, T. 2002. Oxidative stress shortens telomeres. *Trends Biochem Sci*. 27:339-344.

Waguespack, S.G., Hui, S.L., White, K.E., Buckwalter, K.A., and Econs, M.J. 2002. Measurement of tartrate-resistant acid phosphatase and the brain isoenzyme of creatine kinase accurately diagnoses type II autosomal dominant osteopetrosis but does not identify gene carriers. *J Clin Endocrinol Metab*. 87:2212-2217.

Wall, M.J., Wigmore, G., Lopatar, J., Frenguelli, B.G., and Dale, N. 2008. The novel NTPDase inhibitor sodium polyoxotungstate (POM-1) inhibits ATP breakdown but also blocks central synaptic transmission, an action independent of NTPDase inhibition. *Neuropharmacology*. 55:1251-1258.

Wang, F.S., Wang, C.J., Sheen-Chen, S.M., Kuo, Y.R., Chen, R.F., and Yang, K.D. 2002. Superoxide mediates shock wave induction of ERK-dependent osteogenic transcription factor (CBFA1) and mesenchymal cell differentiation toward osteoprogenitors. *J Biol Chem*. 277:10931-10937.

Wang, T.F., Ou, Y., and Guidotti, G. 1998. The transmembrane domains of ectoapyrase (CD39) affect its enzymatic activity and quaternary structure. *J Biol Chem*. 273:24814-24821.

Watanabe, M., and Whitman, M. 1999. The role of transcription factors involved in TGFbeta superfamily signalling during development. *Cell Mol Biol (Noisy-le-grand)*. 45:537-543.

Watts, N.B., and Diab, D.L. 2010. Long-term use of bisphosphonates in osteoporosis. *J Clin Endocrinol Metab*. 95:1555-1565.

Wei, S., Kitaura, H., Zhou, P., Ross, F.P., and Teitelbaum, S.L. 2005. IL-1 mediates TNF-induced osteoclastogenesis. *J Clin Invest*. 115:282-290.

Weitzmann, M.N., Roggia, C., Toraldo, G., Weitzmann, L., and Pacifici, R. 2002. Increased production of IL-7 uncouples bone formation from bone resorption during estrogen deficiency. *J Clin Invest*. 110:1643-1650.

Wesselius, A., Bours, M.J., Agrawal, A., Gartland, A., Dagnelie, P.C., Schwarz, P., and Jorgensen, N.R. 2011. Role of purinergic receptor polymorphisms in human bone. *Front Biosci (Landmark Ed)*. 16:2572-2585.

West, D.C., Sattar, A., and Kumar, S. 1985. A simplified in situ solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. *Anal Biochem*. 147:289-295.

Whitfield, J.F., Morley, P., Ross, V., Preston, E., Soska, M., Barbier, J.R., Isaacs, R.J., Maclean, S., Ohannessian-Barry, L., and Willick, G.E. 1997. The hypotensive actions of osteogenic and nonosteogenic parathyroid hormone fragments. *Calcif Tissue Int*. 60:302-308.

Williams, M., and Jarvis, M.F. 2000. Purinergic and pyrimidinergic receptors as potential drug targets. *Biochem Pharmacol*. 59:1173-1185.

Wright, H.L., McCarthy, H.S., Middleton, J., and Marshall, M.J. 2009. RANK, RANKL and osteoprotegerin in bone biology and disease. *Curr Rev Musculoskelet Med*. 2:56-64.

Wright, T., Rea, S.L., Goode, A., Bennett, A.J., Ratajczak, T., Long, J.E., Searle, M.S., Goldring, C.E., Park, B.K., Copple, I.M., and Layfield, R. 2013. The S349T mutation of SQSTM1 links Keap1/Nrf2 signalling to Paget's disease of bone. *Bone*. 52:699-706.

Wright, W.E., Piatyszek, M.A., Rainey, W.E., Byrd, W., and Shay, J.W. 1996. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet*. 18:173-179.

Xiao, G., Cui, Y., Ducy, P., Karsenty, G., and Franceschi, R.T. 1997. Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. *Mol Endocrinol*. 11:1103-1113.

Xiao, G., Gopalakrishnan, R., Jiang, D., Reith, E., Benson, M.D., and Franceschi, R.T. 2002. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signalling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res*. 17:101-110.

Xiao, G., Jiang, D., Thomas, P., Benson, M.D., Guan, K., Karsenty, G., and Franceschi, R.T. 2000. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem*. 275:4453-4459.

Xu, B., Song, G., and Ju, Y. 2011. Effect of focal adhesion kinase on the regulation of realignment and tenogenic differentiation of human mesenchymal stem cells by mechanical stretch. *Connect Tissue Res*. 52:373-379.

Yang, D., Sun, S., Wang, Z., Zhu, P., Yang, Z., and Zhang, B. 2013. Stromal cell-derived factor-1 receptor CXCR4-overexpressing bone marrow mesenchymal stem cells accelerate wound healing by migrating into skin injury areas. *Cell Reprogram*. 15:206-215.

Yang, S., Wei, D., Wang, D., Phimphilai, M., Krebsbach, P.H., and Franceschi, R.T. 2003. In vitro and in vivo synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. *J Bone Miner Res*. 18:705-715.

Ye, R.D. 2001. Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. *J Leukoc Biol*. 70:839-848.

Yegutkin, G.G. 2008. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta*. 1783:673-694.

Yegutkin, G.G., Henttinen, T., and Jalkanen, S. 2001. Extracellular ATP formation on vascular endothelial cells is mediated by ecto-nucleotide kinase activities via phosphotransfer reactions. *FASEB J*. 15:251-260.

Yegutkin, G.G., Henttinen, T., Samburski, S.S., Spychala, J., and Jalkanen, S. 2002. The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. *Biochem J*. 367:121-128.

You, J., Yellowley, C.E., Donahue, H.J., Zhang, Y., Chen, Q., and Jacobs, C.R. 2000. Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading-induced oscillatory fluid flow. *J Biomech Eng*. 122:387-393.

Yu, X., Collin-Osdoby, P., and Osdoby, P. 2003. SDF-1 increases recruitment of osteoclast precursors by upregulation of matrix metalloproteinase-9 activity. *Connect Tissue Res*. 44:79-84.

Yu, Z., Zhu, T., Li, C., Shi, X., Liu, X., Yang, X., and Sun, H. 2012. Improvement of intertrochanteric bone quality in osteoporotic female rats after injection of polylactic acid-polyglycolic acid copolymer/collagen type I microspheres combined with bone mesenchymal stem cells. *Int Orthop*. 36:2163-2171.

Zaidi, M., Bax, B.E., Shankar, V.S., Moonga, B.S., Simon, B., Alam, A.S., Gaines Das, R.E., Pazianas, M., and Huang, C.L. 1994. Dimensional analysis of osteoclastic bone resorption and the measurement of biologically active calcitonin. *Exp Physiol*. 79:387-399.

Zallone, A. 2006. Direct and indirect estrogen actions on osteoblasts and osteoclasts. *Ann N Y Acad Sci*. 1068:173-179.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y., and Li, L. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*. 425:836-841.

Zheng, L.M., Zychlinsky, A., Liu, C.C., Ojcius, D.M., and Young, J.D. 1991. Extracellular ATP as a trigger for apoptosis or programmed cell death. *J Cell Biol*. 112:279-288.

Zheng, M.H., Fan, Y., Wysocki, S., Wood, D.J., and Papadimitriou, J.M. 1994. Carbonic anhydrase II gene transcript in cultured osteoclasts from neonatal rats: effect of calcitonin. *Cell Tissue Res*. 276:7-13.

Zhou, S., Greenberger, J.S., Epperly, M.W., Goff, J.P., Adler, C., Leboff, M.S., and Glowacki, J. 2008. Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts. *Aging Cell*. 7:335-343.

Zhou, Y.X., Xu, X., Chen, L., Li, C., Brodie, S.G., and Deng, C.X. 2000. A Pro250Arg substitution in mouse Fgfr1 causes increased expression of Cbfa1 and premature fusion of calvarial sutures. *Hum Mol Genet*. 9:2001-2008.

Zimmermann, H. 2000. Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol*. 362:299-309.

Zimmermann, H., Zebisch, M., and Strater, N. 2012. Cellular function and molecular structure of ecto-nucleotidases. *Purinergic Signal*. 8:437-502.

Zippel, N., Limbach, C.A., Ratajski, N., Urban, C., Luparello, C., Pansky, A., Kassack, M.U., and Tobiasch, E. 2012. Purinergic receptors influence the differentiation of human mesenchymal stem cells. *Stem Cells Dev*. 21:884-900.

Ziros, P.G., Gil, A.P., Georgakopoulos, T., Habeos, I., Kletsas, D., Basdra, E.K., and Papavassiliou, A.G. 2002. The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. *J Biol Chem*. 277:23934-23941.